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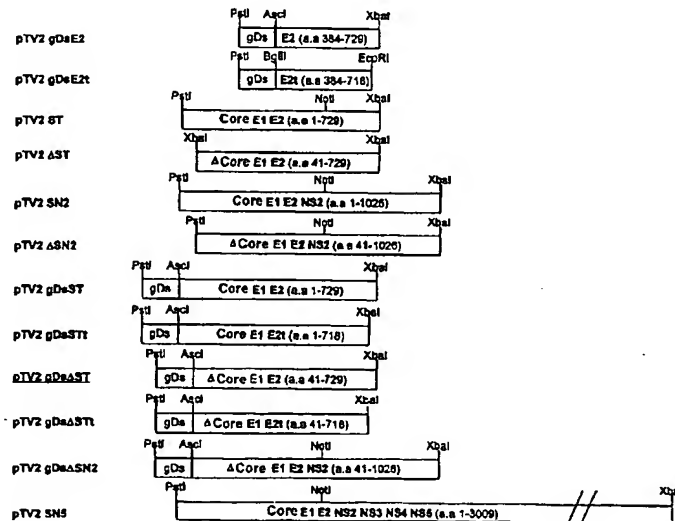
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(54) Title: A VACCINE ENHANCING THE PROTECTIVE IMMUNITY TO HEPATITIS C VIRUS USING PLASMID DNA
AND RECOMBINANT ADENOVIRUS



(57) Abstract: The present invention relates to a vaccine enhancing the protective immunity to Hepatitis C virus using plasmid DNA recombinant adenovirus, more particularly to a vaccine consisting of Δ core-E1-E2 expressing DNA vaccine, nonstructural protein NS3 and NS4 expressing DNA vaccine, nonstructural protein NS5 expressing DNA vaccine and recombinant adenovirus vaccine, and method for administration of the vaccine by priming with the DNA vaccines described above and boosting with the recombinant adenovirus vaccine thereby enhancing the protective immunity to Hepatitis C virus.

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A VACCINE ENHANCING THE PROTECTIVE IMMUNITY
TO HEPATITIS C VIRUS USING PLASMID DNA AND
RECOMBINANT ADENOVIRUS

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FIELD OF THE INVENTION

The present invention relates to a DNA vaccine,
a recombinant adenovirus vaccine, and a method for
administration of those vaccines enhancing the
protective immunity to hepatitis C virus, more
10 particularly, a DNA vaccine prepared to induce the
optimum cellular immune response through an antigen
engineering, a recombinant adenovirus vaccine, and a
vaccination method enhancing the protective immunity
against HCV infection by regulating the same.

15

BACKGROUND

HCV (hepatitis C virus) was first found as a
major pathogenic microorganism of non-A and non-B
(NANB) type hepatitis by Chiron Company in U.S.A. in
20 1989 (Choo, *Science*, 1989, 244:359-362). The
development of HCV diagnostic kit facilitated the
research on the actual state of HCV infection. In
result, it has been reported that 170 million people
have been infected with HCV all over the world, and

1.5% of the Korean population are HCV carriers. Once infected with HCV, 85% of carriers pass into chronic state, which is very high ratio, and chronic HCV leads to hepatocirrhosis and liver cancer (Bisceglie, *Hepatology*, 1997, 26: 345), recognizing HCV to be a cause of very threatening disease. However, an effective treatment agent and a vaccine for HCV have not been developed yet.

10 The states of immunity of both patients recovering from HCV infection and those passing into chronic hepatitis were clinically investigated. While HCV specific cellular immune response was observed in the patients in recovery, the response was not detected in patients switched over chronic hepatitis (Rehermann, *J. Virol.*, 1996, 70:7092; Lechner, *J. Immunol.*, 2000, 30:2479). Especially, CD4+ Th1 immune response has been known to be closely related to the protection and the recovery from HCV infection (Rosen, *Hepatology*, 2002, 35:190; Sarih, *Immunol. lett.*, 2000, 74:117; Diepolder, *J. Mol. Med.*, 1996, 74:538). According to the research by Pape group, the conditions of patients infected with acute hepatitis are classified into three types; 1) strong CD4+ Th1 immune response is observed in the patients

group free from the virus, 2) CD4+ T cell immune response is not observed in the patients group switched over chronic hepatitis, and 3) CD4+ T cell immune response is seen at early stage when the virus is in control, but as the immune response weakens, HCV returns (Gerlach, *Gastroenterology*, 1999, 117:933). The above studies support the importance of CD4+ Th1 immune response in the control or elimination of HCV. It was also reported from the studies using a chimpanzee, a unique test animal in which HCV infection and replication are allowed, that rather cellular immunity than humoral immunity played an important role in recovery from HCV infection (Cooper, *Immunity*, 1999, 10:439). Such results suggest that a strong multi-epitope specific Th1 immunity is required for the prevention and the treatment of HCV. Thus, the development of a vaccine for the prevention and the treatment of HCV are now focused on inducing the optimum Th1 cellular immune response.

A subunit protein vaccine was the first HCV preventive vaccine developed by using surface proteins of HCV, envelop 1 and 2 (E1, E2). HCV E1E2 is surface protein of the virus binding to a receptor

of a host cell as being infected. If the host cell has a neutralizing antibody against the protein, HCV infection can be prevented. E1E2 subunit vaccine was tried in chimpanzees, precisely, homologous challenge was tried with 10 CID₅₀ of infection dose. As a result, HCV infection was successfully prevented in 5 out of 7 chimpanzees, and even after being infected, the rest 2 chimpanzees did not progress to chronic hepatitis (Choo, *Proc. Natl. Acad. Sci. USA*, 1994, 91:1294). That was the first report on HCV preventive vaccine. Even though the effect of the vaccine was proved in chimpanzees, the study has limitations as follows. First, small dose of challenging HCV (10 CID₅₀) was inoculated at the peak time of antibody response. Generally, a vaccine has to protect against viral infection by memory response in any circumstances. In the above report, though, antibody response decreased rapidly after the challenging time point. Thus, it is doubtful that the similar protective effect can be achieved when challenge is done after the antibody response decreased. Second, the vaccine was effective against homologous challenge but not heterologous challenge. Considering HCV has at least 6 major genotypes and great numbers of subtypes (Bukh, 1995, *Semin Liver*

Dis 15: 41-63), a vaccine must have preventive effect against heterologous challenge at least with in the same genotype. Third, the protective effect of the vaccine depended on not cellular immune response but antibody response. Recently, along with the reports announcing the importance of cellular immunity for the protective immunity to HCV, reports asserting the limitation of antibody response have been made (Cooper, 1999, *Immunity*, 10: 439; Esumi, 2002, Vaccine, 20:3095-3103). So, the protein vaccine depending on antibody response alone as a defense mechanism is questionable. Therefore, attempts to induce cellular immunity with DNA vaccine have been made, and as an example, immunity was induced by DNA expressing E2, a surface protein of HCV, resulting in the protective effect against challenge with 100 CID₅₀ of homologous monoclonal HCV (Forns X., *Hepatology*, 2000, 32(3): 618-25). It was meaningful as the first report on the preventive vaccine using DNA inducing cellular immunity in chimpanzees, but still had problems, too. First, the challenge was performed at the peak time of immune response. Second, monoclonal HCV challenging inoculum was used for the challenge. Like HCV or HIV (human immunodeficiency virus), the virus that uses error-

prone RNA dependent RNA polymerase for replication is characterized by producing numbers of quasispecies. Such variety of quasispecies plays an important role for HCV to establish chronic infection (Farci P, 2000, *Science*, 288:339). According to a recent study, HCV can be produced by intrahepatic injection of HCV RNA into chimpanzee (Kolykhalov, 1997, *Science*, 277: 570-4). The recovered HCV was infectious and used in the study by Forns et al. Since the monoclonal HCV cannot provide a variety of quasispecies which exist in reality, whether the protective effect is still the same when a real infectious HCV attacks is doubtful. Lastly, based on the observation on the immune response and the course of viral infection, it was unclear that the above result was obtained by the immune response induced by the vaccine. That is, immunological evidence which distinguish the case from the natural recovery (about 50%) was not enough to support the protective effect of the vaccine.

A DNA vaccine is superior to a protein vaccine in inducing cellular immune response. Since the antigen of DNA vaccine is expressed in host cells, it can induce humoral immunity with almost native conformation, and even simultaneously induce CD8+ T

cell response, a kind of cellular immunity that a protein vaccine cannot induce, so that a DNA vaccine enhances the protective immunity to the maximum and easily induces Th1 immune response simply through the intramuscular injection (Pertmer, *J. Virol.*, 1996, 70:6119). Unlike an inactivated vaccine or a killed vaccine, a DNA vaccine uses only a specific region of the virus as an antigen, causing fewer side effects. In addition, it is easy to store and convey a DNA vaccine, and the purification of the plasmid is also simple, comparing to other vaccines. The safety of a DNA vaccine was approved by Food and Drug Administration (FDA), USA, so that an AIDS DNA vaccine was allowed for clinical study in 1996. After the successful induction of immune response in small animal model, DNA immunization has been tried in many large animal models. Unfortunately, protective immunity against a highly pathogenic virus infection was not secured in large animal models. In experiments with HCV DNA vaccine in chimpanzees, the antibody and cellular immunity induced by DNA vaccine alone were so weak (Forns, *Hepatology*, 2000, 32:618) that another type of vaccine capable of inducing a strong cellular immunity was required.

Even though the limitation of a DNA vaccine has been widely known, the merit of the vaccine that can prime delicate immune response and Th1 immunity encouraged the study to overcome the limitation by combining with other boosting method. Previous reports suggest that the effect of the vaccine is greatly enhanced when boosted with a recombinant protein or an attenuated recombinant virus after priming with DNA. Such attempts were successful in small animals by inducing protective immunity against challenges (Song, *J. Virol.*, 2000, 74:2920; Hanke, 1998, *Vaccine*, 16: 439-45; Sedegah, 1998, *Proc. Natl. Acad. Sci. USA*, 95: 7648-53; Schneider, 1998, *Nat Med*, 4: 397-402), and so was in Primates (Kent, 1998, *J Virol*, 72: 10180-8; Robinson, 1999, *Nat Med*, 5: 526-34; Amara, 2001, *Science*, 292: 69-74). Yet, there has been no report on the protective effect of DNA prime and adenovirus boosting regimen against hepatitis C virus infection.

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Adenovirus has been proved safe and widely used as a vector for gene therapy. It was also proved to be very useful as a vaccine since it could induce strong humoral, cellular immune responses in various animal models (Natuk, *Proc. Natl. Acad. Sci. USA*,

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1992, 89(16): 7777; Bruce, J. *Gen. Virol.*, 1999, 80:2621). An earlier report demonstrated that the induced immune response was maintained for a long time after single injection of a replication-defective adenovirus (Juillard, *Eur. J. Immunol.*, 1995, 25:3467). The antibody response and cellular immune response were also induced in small animal models by immunization with recombinant adenovirus expressing HCV structural gene (Makimura, *Vaccine*, 1996, 14:28; Bruna-Romero, *Hepatology*, 1997, 25:470; Seong, *Vaccine*, 2001, 19:2955). However, the induced Th1 immunity including CTL response was not compared in parallel with a DNA vaccine. Under this circumstance, DNA priming and adenovirus boosting regimen was tested in monkey model and proved its potential as a next generation vaccine regimen (Sullivan, 2000, *Nature*, 408: 605-9; Shiver, 2002, *Nature*, 415: 331-5).

20 The present inventors developed a DNA vaccine which induces optimal level of cellular immune response to hepatitis C virus through antigen engineering and confirmed that optimal Th1 immune response was induced by DNA priming and adenovirus
25 boosting regimen. Finally, the present inventors

proved in chimpanzee study that the vaccine regimen of the invention could induce the protective immunity against hepatitis C virus infection.

5 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In order to achieve an object of the present invention, this invention provides a DNA vaccine including plasmid containing 2-6 kb, more preferable, 2-4 kb of the total antigen gene of hepatitis C virus, more precisely, provides a DNA vaccine including the
10 1st plasmid containing core, E1 and E2 genes, the 2nd plasmid containing NS3 and NS4 genes, and the 3rd plasmid containing NS5 gene.

The present invention also provides a DNA
15 vaccine characterized by having the 1st plasmid containing core, in which 35-40 amino acids of amino-terminal region are eliminated, and a DNA vaccine characterized by having transmembrane domain of E2 in the 1st plasmid above.

20 The present invention further provides a DNA vaccine in which the 1st plasmid has a base sequence represented by SEQ. ID. No 50, particularly, a DNA vaccine whose 1st plasmid is pGX10 gDsΔ ST (Accession No: KCCM 10415); a DNA vaccine in which the 2nd

plasmid includes a base sequence represented by SEQ. ID. No 51, particularly, a DNA vaccine whose 2nd plasmid is pGX10 NS34 (Accession No: KCCM 10417); a DNA vaccine in which the 3rd plasmid contains a base sequence represented by SEQ. ID. No 52, particularly, a DNA vaccine whose 3rd plasmid is pGX10 NS5 (Accession No: KCCM 10416).

The present invention also provides a DNA vaccine in which the 1st plasmid includes a base sequence represented by SEQ. ID. No 50, the 2nd plasmid has a base sequence represented by SEQ. ID. No 51, and the 3rd plasmid contains a base sequence represented by SEQ. ID. No 52; more precisely, a DNA vaccine characterized by that the 1st plasmid is pGX10 gDsΔ ST (Accession No: KCCM 10415), the 2nd plasmid is pGX10 NS34 (Accession No: KCCM 10417), and the 3rd plasmid is pGX10 NS5 (Accession No: KCCM 10416), and a DNA vaccine further supplemented with pGX10 hIL-12m.

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In order to achieve another object of the invention, the present invention provides a recombinant adenovirus vaccine including adenovirus containing 2-6 kb, preferably 2-4 kb, of total antigen gene of hepatitis C virus, more precisely, a

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recombinant adenovirus vaccine characterized by having all of the 1st adenovirus containing core, E1 and E2 genes, the 2nd adenovirus containing NS3 gene and NS4 gene, and the 3rd adenovirus containing NS5 gene.

The present invention also provides a recombinant adenovirus vaccine in which 35-40 amino acids of amino-terminal region of core are eliminated in the 1st adenovirus, and a recombinant adenovirus vaccine containing transmembrane domain of E2 in the 1st adenovirus above.

The present invention further provides a recombinant adenovirus vaccine whose 1st adenovirus has a base sequence represented by SEQ. ID. No 50, precisely, the 1st adenovirus is rAd gDsΔ ST (Accession No: KCCM 10418); a recombinant adenovirus vaccine whose 2nd adenovirus has a base sequence represented by SEQ. ID. No 54, precisely, the 2nd adenovirus is rAd gDs NS34 (Accession No: KCCM 10420); and a recombinant adenovirus vaccine whose 3rd adenovirus has a base sequence represented by SEQ. ID. No 52, precisely, the 3rd adenovirus is rAd NS5 (Accession No: KCCM 10419).

The present invention also provides a recombinant adenovirus vaccine composed of the 1st

adenovirus having a base sequence represented by SEQ.
ID. No 50, the 2nd adenovirus having a base sequence
represented by SEQ. ID. No 54, and the 3rd adenovirus
having a base sequence represented by SEQ. ID. No 52;
5 more particularly, a recombinant adenovirus vaccine
in which the 1st adenovirus is rAd gDsΔ ST (Accession
No: KCCM 10418), the 2nd adenovirus is rAd gDs NS34
(Accession No: KCCM 10420) and the 3rd adenovirus is
rAd NS5 (Accession No: KCCM 10419).

10

In order to achieve other object of the
invention, the present invention provides a vaccine
administrating method characterized by enhancing the
protective immunity to HCV by boosting with the above
15 adenovirus vaccine after priming with the DNA vaccine
2-5 times.

The present invention also provides a method to
enhance the protective immunity to HCV by increasing
CD4+ Th1 immune response by boosting with a
20 recombinant adenovirus vaccine after priming with a
DNA vaccine.

The present invention further provides a method
for the prevention and the treatment of hepatitis C,
which is characterized by boosting with a recombinant
25 adenovirus vaccine after priming with a DNA vaccine.

The term "DNA vaccine" herein means a plasmid containing a gene coding a protein that works as an antigen or a substance containing the said plasmid and pharmaceutical components that are generally added for the vaccine preparation.

The term "priming" means primary antigen-stimulation (sensitization), and the term "boosting" means additional immunity after primary antigen-stimulation.

Hereinafter, the present invention is described in detail.

The whole gene sequence of HCV is divided into a structural protein region (Core-E1-E2) gene and two other non-structural protein region (NS3-NS4 and NS5) genes, and the present invention provides individual plasmids (or recombinant adenoviruses) containing each of the above genes.

RNA virus such as HCV or HIV escapes from host immune surveillance by generating diverse quasispecies since the acute phase of infection (Shimizu YK et al, 1994, *J Virol*, 68:1494-500; Weiner A et al, 1995, *Proc. Natl. Acad. Sci. USA*, 92:2755;

Wyatt CA et al, 1998, *J Virol*, 72:1725; Erickson AL
et al, 2001, *Immunity*, 15:883), which was known as a
major mechanism for the virus to persist (Farci P et
al, 2000, *Science*, 288:339). In order to protect
5 against the virus infection, multi-epitope specific
cellular immunity has to be induced simultaneously
(Cooper, 1999, *Immunity*, 10:439; Lechmann, 2000,
Semin Liver Dis 20(2): 211), which required the
vaccine to include as long part of viral gene as
10 possible. For this, it is necessary to optimize
insert length in a DNA vaccine (or in a recombinant
adenovirus vaccine).

For example, when ten kinds of HCV proteins are
individually expressed by separate plasmids (or
15 adenoviruses), a vector backbone takes huge part in
DNA vaccine, comparing to the length of a gene taken
by an antigen. Therefore, large amount of DNA is
required for sufficient level of expression of the
antigen. Inefficiency is another problem in the
20 production of a vaccine or the experiments of
toxicity aiming at clinical researches. On the
contrary, if a whole HCV gene is expressed in a
plasmid (or adenovirus), the expression during
transcription and translation becomes inefficient,
25 which not only is inadequate to induce an effective

cellular immunity but also affects the stability of DNA during the production and purification processes. In order to overcome the potential problems, which range of insert length in a DNA vaccine can efficiently induce cellular immunity should be scrutinized. The strategy is to find out a DNA vaccine with proper insert length that possesses as large part of a gene as possible and induces as strong cellular immunity as well as a DNA vaccine that expresses whole HCV gene. As a result, a plasmid (or adenovirus) including 2-6 kb of total HCV gene was proved to be very effective, and preferably, a plasmid (or adenovirus) including 2-4 kb, more preferably, a vaccine including three individual plasmids (or adenoviruses) containing one structural protein domain (Core-E1-E2) and two non-structural protein domains (NS3-NS4 and NS5), respectively, was developed. As of today, at least six major genotypes of HCV including HCV-1, HCV-J, etc., have been reported. Structural region of HCV composed of core, E1 and E2, and non-structural region composed of NS2, NS3, NS4 and NS5. 2-6 kb size HCV gene included in the plasmid (or adenovirus) of the invention is not limited in the genotypes.

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The present invention also provides a plasmid (or recombinant adenovirus) in which 35-40 amino acids of amino-terminal region of core are eliminated, and genes E1 and E2 are included. At this time, the number of amino acids for the elimination is preferably 40, and E2 preferably keeps transmembrane domain.

Core of HCV is the region that has the highest homology with many other types, making it the best target for the development of a vaccine against heterologous infection (Shirai M. 1994, *J Virol*, 68:3334-42; Inchauspe G., 1995, *J Virol*, 69:5798-5805; Wands JR, 1996, *Hepatology*, 24:14-20; Geissler M., 1997, *J Immunol*, 158:1231-7; Arichi T, 2000, *Proc. Natl. Acad. Sci. USA*, 97:297-302; Polakos NK., 2001, *J Immunol*, 166(5):3589-3598). However, it was reported that HCV core has immunosuppressive function (Large, 1999 *J Immunol* 162:931-8, Lee, *Virology*, 2001, 279:271). Thus, the present inventors developed a vaccine without core's immunosuppressive function by removing 35-40 amino acids of amino-terminal region of core, and especially, the elimination of 40 amino acids was confirmed to be more effective.

In the case of using the envelope protein of

HCV as a vaccine, a DNA without transmembrane domain of E2 was used to induce extracellular secretion for the induction of antibody response. Yet, considering the report that the antibody response might not provide the protective immunity against HCV infection (Cooper, 1999, *Immunity*, 10:439; Esumi, 2002, *Vaccine*, 20: 3095-3103), the present inventors developed the said DNA vaccine (or recombinant adenovirus vaccine) keeping transmembrane domain of E2 based on the
10 result that the DNA can induce cellular immunity better than the DNA without transmembrane domain.

The present inventors developed plasmids pGX10 gDs Δ ST (Accession No: KCCM 10415), pGX10 NS34
15 (Accession No: KCCM 10417) and pGX10 NS5 (Accession No: KCCM 10416), and named the mixture DNA vaccine HC102 (FIG. 2). pGX10 gDs Δ ST includes core gene coding core protein of HCV, and E1 and E2, both encoding envelope protein, pGX10 NS34 includes genes
20 corresponding to non-structural proteins 3 and 4 (NS34), and pGX10 NS5 includes a gene corresponding to structural protein 5 (NS5). IL-12 mutant DNA (pGX10 mIL-12m) (Ha, 2002, *Nat Biotechnol*, 20: 381-6), which is known to enhance the immunogenicity of HCV
25 DNA vaccine in small animal model, could be further

included in HC102, and the DNA vaccine containing pGX10 hIL-12m was named HC103 (FIG. 3).

The present inventors developed a DNA vaccine vector included in AIDS DNA vaccine, which was named pGX10. As shown in FIG. 2, the vector pGX10 of the present invention consists of simian virus 40 origin (SV40 ori), cytomegalovirus (CMV) promoter/enhancer sequence, adenovirus tripartite leader sequence (TPL), multi-cloning site (MCS), simian virus 40 polyA sequence (SV40PA), simian virus 40 enhancer sequence (SV40Eh), and additionally ColE1 Ori and kanamycin-resistant gene (KanR) which enable a plasmid to proliferate in *E. coli*. It is a novel vector in 3.6 kb size including several specific restriction regions. The vector pGX10 was prepared based on pTX (Lee, Vaccine 17:473-9, (1999)) that was reported previously by the present inventors. Precisely, vector pTV2, already used as a DNA vaccine vector in the studies in small animals (Lee, J Virol. 72,8430(1998); Cho, Vaccine 17,1136(1999)), was used as a starting vector and the preparation process was as informed. The resultant novel vector developed by the present inventors was deposited at KCTC (Accession No: 10212BP). By the way, the promoter types and the sorts and sizes of glycoprotein signal

sequences can be changed depending on the object of the examples of the invention. For example, RSV promoter, a viral promoter, and EF1, MCK (muscle specific promoter) and LCK (T cell specific promoter),
5 cellular promoters, can be selected according to circumstances, and glycoprotein can also be substituted with VZV (varicella zoster virus) gB, HCMV (human cytomegalovirus) gH, gL, gO, VSV (vesicular stomatitis virus) G protein, rotavirus
10 outer capsid glycoprotein, and VP7.

In order to prevent the degradation of DNA and to promote transduction into cells, the plasmid of the invention preferably forms a complex with cationic lipid like DMRIE/DOPE. The plasmid DNA can
15 be regarded as a drug substance and can be produced in a well-characterized *E. coli* host. Once enters into cells, a recombinant gene of the plasmid is expressed, resulting in the production of a gene product having a biochemical activity. For the
20 production of a drug substance, the plasmid DNA is mixed with lipids DMRIE and DOPE in an injection vehicle. DMRIE/DOPE lipid complex has a positive electric charge overall, so that it can combine with DNA having a negative electric charge. The resultant
25 plasmid DNA-lipid complex is directly injected into

an object.

It is a common knowledge for the people in this field that the said plasmid of the present invention is presented as a preferable example, having no
5 intention to limit the present invention therein. And thus, every expression vector that is suitable for the conveyance of the subjects and the acquisition of the production, and has all the merits mentioned above can be included in the category of
10 the present invention.

The present invention further provides individual recombinant adenoviruses each including structural genes of HCV, core, E1 and E2, non-
15 structural genes, NS3 and NS4, and another non-structural gene, NS5, respectively (FIG. 4). At this time, the recombinant adenovirus includes the same HCV gene structure and territory as the above plasmid expressing HCV gene.

20 The recombinant adenovirus of the present invention is characterized by having the said HCV genes in addition to the basic structure of the general adenovirus. In order to prepare HCV adenovirus vaccine, the present inventors developed a
25 recombinant adenovirus by inserting HCV antigen genes

'gDsΔ ST', 'gDsNS34' and 'NS5' in a replication defective adenovirus vector without E1 gene essential for the virus replication, and each was named 'rAd gDsΔ ST' (Accession No: KCCM 10418), 'rAd gDsNS34' (Accession No: KCCM 10420) and 'rAd NS5' (Accession No: KCCM 10419), and the vaccine including all of them was named rAd-HC102.

The "immunologically effective" dosage of the vaccine compound used in this invention means the amount of single or multiple administrations that is effective for the prevention or the treatment. The specific dosage depends on health and body condition of an individual, classified groups (for example: human, nonhuman primates, primates, etc.), the condition of immune system, the formulations of vaccine, the decision of a doctor in charge, the pathway of HCV infection and other relating factors. The dosage can be decided by general trial, comparatively having a wide range. Generally, the dosage of the vaccine of the invention ranges from 1 μ g to 20 mg per administration. The regulation of dosage according to the mentioned method or other standard way for the maximum effect is also regarded as natural to the people in this field.

The vaccine can be given by single or preferably multi-administration. DNA priming-rAd boosting immunization is preferably used for the multi-administration of a DNA vaccine and a recombinant adenovirus vaccine.

The vaccine is optionally compounded of vegetable oil or its emulsion; surfactant such as hexadecylamine, octadecyl amino acid ester, octadecylamine, lysolecithin, dimethyl-dioctadecylammonium bromide, N,N-dioctadecyl-N'-N'-bis (2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol and fluoric polyol; polyamine such as pyran, dextran sulfate, poly IC and carbopol; peptide such as muramyl dipeptide, dimethylglycine and turpethsin; immunostimulating complex; oil emulsion; lipopolysaccharide such as MPLR (3-O-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, Montana) and mineral gel. The plasmid DNA or the recombinant adenovirus of the present invention can be further supplemented with liposome, cochleates, biodegradable polymer such as poly-lactide, poly-glycolide and poly-lactide-co-glycolide, or ISCOMS (immunostimulating complex), and with additional activating factors, as well. The plasmid of the

present invention can be administered along with bacterial toxin and its attenuated derivatives.

The vaccine can be administered to human or animals through various routes including parenteral, intra-arterial, intracutaneous, transdermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal administration (not always limited thereto). The dosage of an antigen used for the vaccine varies upon the homogeneity of the antigen. It depends on the decision made by the people in this field to regulate and manipulate the set amount of the vaccine used together with a general carrier antigen for the vaccine of the present invention.

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The present invention also provides a method for the administration of a vaccine characterized by enhancing the protective immunity to HCV by boosting with a recombinant adenovirus vaccine after priming with a DNA vaccine.

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In order to enhance the protective immunity, there are four possible ways: only a DNA vaccine is injected to an object, only a recombinant adenovirus vaccine is injected, priming with a DNA vaccine is followed by boosting with a recombinant adenovirus

25

vaccine, or priming with a recombinant adenovirus vaccine is followed by boosting with a DNA vaccine. However, in order to get the optimum effect of the immunity to HCV, boosting with a recombinant adenovirus vaccine after priming with a DNA vaccine is preferred. The priming frequency is preferably 2-5 times, and particularly, three times of DNA priming and the following one performance of adenovirus boosting was proved to be more effective in earlier experiments with chimpanzees.

Precisely, in order to get the optimum protective immunity to HCV, three times of priming with the DNA vaccine containing all of pGX10 gDsΔ ST (Accession No: KCCM 10415), pGX10 NS34 (Accession No: KCCM 10417), and pGX10 NS5 (Accession No: KCCM 10416), or the DNA vaccine supplemented with pGX10 hIL-12m to the above ought to be preceded by the one time boosting with the recombinant adenovirus vaccine containing all of rAd gDsΔ ST (Accession No: KCCM 10418), rAd gDs NS34 (Accession No: KCCM 10420), and rAd NS5 (Accession No: KCCM 10419).

The present invention further provides a method for the prevention or the treatment of hepatitis C by administering a DNA vaccine and/or a recombinant

adenovirus vaccine.

The method for the prevention or the treatment of hepatitis C with a DNA vaccine and/or a recombinant adenovirus vaccine preferably uses DNA-priming-rAd boosting method, which is effective in inducing both CD4+ Th1 immune response and cytotoxic T lymphocyte response. Especially, DNA priming-rAd-boosting method is particularly effective in inducing CD4+ Th1 immune response that has been known to play an important role in eliminating HCV and recovery from illness. Thus, the said method can be effectively used for the prevention and the treatment of HCV.

In order to confirm the antigen expression by the HCV DNA vaccine and the recombinant adenovirus vaccine prepared in this invention, COS-7 animal cells (ATCC CRL-1651) were transfected with vectors pGX10 gDsΔ ST, pGX10 NS34 and pGX10 NS5, in addition to a control vector pGX10. The transfected cells were harvested to confirm the expression of core, E2, NS3, NS4, and NS5 by Western blot analysis using antigen-specific antibodies. COS-7 cells were also transfected with rAd gDsΔ ST, rAd gDsNS34, and rAd NS5, in addition to the control virus rAd-mock

produced and purified by using 293A cells. Then, the transfected cells were harvested to confirm the expression of the mentioned antigens by Western blot analysis also using the said antibodies.

5 As a result, unlike in the control vector pGX10 and control virus rAd-mock infected cells, HCV core, E2, NS3, NS4 and NS5 proteins were expressed in animal cells cultured after the injection with pGX10 gDsΔ ST, pGX10 NS34 and pGX10 NS5 or rAd gDsΔ ST, rAd
10 gDsNS34 and rAd NS5. So, the plasmid and the recombinant adenovirus of the present invention were confirmed to express HCV proteins (FIG. 5 - FIG. 8).

The present inventors performed experiments
15 with animal samples using pGX10 gDsΔ ST and pGX10 mIL-12 mutant as DNA vaccines and using rAd gDsΔ ST as a recombinant adenovirus vaccine. In order to investigate what vaccine can best induce CD4+ Th1 immune response which has been known to be most
20 important for the elimination of HCV and the recovery from illness, CD4+ T cells were selected and used for IFN-γ and IL-4 ELISPOT (Enzyme-linked immunospot) assays. Cytokine IFN-γ is the representative Th1 cytokine that is secreted in activated T-cells. On
25 the contrary, cytokine IL-4 is the representative Th2

cytokine that induces B-cell differentiation and Th2 immune response. ELISPOT was performed to quantify the cells secreting those cytokines. As a result, DNA priming-rAd boosting method with HCV E2 and core protein antigens was proved to make CD4+ T cells produce IFN- γ most, comparing to other methods (G2 in FIG. 13A and FIG. 13B, $p < 0.001$). While CD4+ T cells producing E2 specific IFN- γ were hardly generated when just a DNA vaccine was treated twice (FIG. 13A, G1), CD4+ T cells producing core protein specific IFN- γ were generated as much as the group treated with rAd twice (FIG. 13B, G1). That was because core protein antigen itself, unlike E2, could effectively induce CD4+ T cell immune response with the treatment of a DNA vaccine. alone. By contrast, there was barely detectable core- or E2-specific IL-4 ELISPOT response in all groups (FIG. 13C and FIG. 13D), supporting the idea that a DNA vaccine and an adenovirus vaccine could induce Th1 CD4+ T cell immune response rather than Th2 immune response. Taken together, the present inventors confirmed that the method of the invention, in which the immunization of a DNA vaccine followed by recombinant adenovirus boosting, induced better CD4+ Th1 immune response than the immunization with an

adenovirus twice or adenovirus followed by the boosting with a DNA vaccine.

HCV E2-specific ^{51}Cr release assay was also performed to investigate cytotoxic T lymphocyte response, in which adenovirus twice and DNA priming-rAd boosting method were both confirmed to be very efficient, without a significant difference between the two regimens (see FIG. 14, G2 and G3). Even though rAd priming-DNA boosting method and the twice injection of DNA showed antigen-specific cytotoxic T lymphocyte response, those were not so much effective as the above two methods (see FIG. 14, G1 and G4). So, the DNA priming-rAd boosting method was confirmed to be the most effective way to induce cytotoxic T lymphocyte response.

The present inventors proved the effect of the vaccines of the invention by investigating whether the vaccines could inhibit the replication of the infectious HCV in chimpanzees, the only reliable animal susceptible to HCV infection, after inducing protective immunity.

Precisely, the level of cellular immunity induced in chimpanzees by using a DNA vaccine and a

recombinant adenovirus vaccine was investigated. And also, after challenging with the infectious hepatitis C virus, how the vaccine-induced immunity could effectively prevent the replication of hepatitis C virus was examined.

The present inventors attempted to enhance cellular immunity by boosting with a recombinant adenovirus after priming with HCV three times. At that time, hIL-12 mutant proved to promote memory immune response of cellular immunity was simultaneously injected for comparison. HC102, a mixture DNA vaccine consisted of pGX10 gDs Δ ST, pGX10 NS34 and pGX10 NS5, was administered to the experimental group 1, and HC103 prepared by adding pGX10 hIL-12m DNA to HC102 was administered to the experimental group 2. After injecting a DNA vaccine three times, boosting with the recombinant adenovirus 'rAd-HC102' expressing the same region of HCV as HC102 was performed on the 30th week after last DNA injection, and cellular immunity was investigated after two weeks from then. Cellular immunity was analyzed by IFN- γ ELISPOT, which has been known as the most sensitive method for cellular immunity. As a result, 4 out of 6 chimpanzees administered with the vaccine showed very strong immune response but

the remaining 2 chimpanzees showed marginal response (see FIG. 16). No difference was observed between the experimental group 1 and group 2. One of the control group chimpanzees (#404) treated with nothing, 5 though, showed relatively strong IFN- γ ELISPOT response. That meant the chimpanzee was exposed with HCV, supported by the unpublished report from New York Blood Center. In order to be selected for control group, a chimpanzee should be free from 10 previous history of viremia and HCV-specific antibody response. All the chimpanzees here in the control group met the standard, but if they had been exposed to subinfectious dose of HCV, cellular immunity could be induced without viremia (Shata MT, *Virology* 2003, 15 314:601-16).

While FIG. 16 represents combined activity of and CD8+ T cells, FIG. 17 and FIG. 18 show the activity of CD4+ T cells only. As an index of Th1 immune response, the amount of IFN- γ secreted by 20 CD4+ T cells was measured. As a result, comparatively large amount of IFN- γ was detected in the experimental group 1. In the experimental group 2, small amount of IFN- γ was detected, which was, though, higher than that of the control group. 25 Another CD4+ T cell response, T cell proliferate

response, was examined as well. As a result, the level of the response was higher in the experimental group 1 than in the group 2, and was barely detectable in the control group. At that time, one
5 of the control group chimpanzees (#406) showed the similar proliferate response to the vaccine-injected chimpanzees, suggesting its previous exposure of subinfectious dose of HCV.

10 In order to investigate the effect of cellular immune response on the control of the viral replication, challenge with 100 CID₅₀ infectious HCV-bk, different from the vaccine strain, was performed. The challenge condition was set to evaluate the
15 vaccine regimen according to the following issues. First, it had to be confirmed that the cellular immune response induced by the vaccine regimen of the present invention could exert long-term protective effect. Even after the vaccination, the moment of
20 the virus infection is unknown. So, to be an effective vaccine, it should keep its protective immunity for a long while after the vaccination. While the challenges have been generally done on the 2nd - 3rd week after the last vaccination in previous
25 reports (Choo, Proc. Natl. Acad. Sci. USA, 1994,

91:1294; Forns X., *Hepatology*, 2000, 32(3): 618-25),
it was performed on the 12th week in this invention,
at which the immunity usually decline, to investigate
whether the lowered immune response could prevent the
5 HCV infection. Second, it ought to be confirmed that
the cellular immunity induced by the regimen of the
present invention could protect against high dose of
HCV infection, for which not only a vigorous
cellular immunity should be induced but also
10 surveillance over diverse quasispecies should be
established, since the diversity of quasispecies
would increase as dose of challenging inoculum
increases (Wyatt CA 1998 *J Virol* 72:1725). For that
purpose, the dose of the challenging virus was
15 adjusted to 100 CID₅₀. Lastly, it had to be confirmed
that the cellular immunity induced by the method of
the present invention could provide cross protection
against different strain of HCV within the same
genotype. While 5 - 6 logs of viral load was observed
20 in the control group on the 2nd week after the
infection, the virus was hardly detectable in 5 out
of 6 vaccinated chimpanzees. The viral loads in two
chimpanzees (#400, # 381) were borderline positive,
which were determined as negative in repeated
25 experiments for the confirmation, suggesting that the

viral loads were slightly below the detection or, if above, very low level compared with the control group. According to the report on 38 chimpanzees challenged with HCV, the peak viral load was $10^{5.74}$ - $10^{5.82}$ (Prince AM, 2002, *9th International Meeting on HCV and Related Viruses*, P-259). Comparing the value with the conventional statistics or that of the control group of the present invention, the protective immunity induced by the present invention was proved to be protective against acute phase of HCV infection in chimpanzees.

BRIEF DESCRIPTION OF THE DRAWINGS

The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

FIG. 1 is a schematic diagram showing the plasmids used for the small animal experiments in this invention (the numbers in parentheses present amino acid ranges of HCV antigen, which are equal to those in FIG. 2 - FIG. 4),

gDs : Signal sequence of Herpes simplex virus (HSV) glycoprotein D (gD)

FIG. 2 is a schematic diagram showing the plasmid 'HC102' used for chimpanzee experiments of the present invention,

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FIG. 3 is a schematic diagram showing the plasmid 'HC103' used for chimpanzee experiments of the present invention,

10

FIG. 4 is a schematic diagram showing the recombinant adenovirus (rAd) used in the present invention,

15

FIG. 5 is a set of electrophoresis photographs showing whether HCV antigens are expressed in COS-7 cell line, by the plasmid used for the small animal samples in the present invention, which was confirmed by Western blot analysis using monoclonal antibodies to HCV E2,

20

FIG. 6 is an electrophoresis photograph showing the expression of core of the plasmid used for the small animal experiments of the present invention in COS-7 cell line, and the elimination of N terminal 40 amino acids which was confirmed by Western blot

25

analysis using polyclonal antibodies against core,

FIG. 7 is a set of electrophoresis photographs showing the expression of the plasmids used in chimpanzee experiments of the present invention in COS-7 cell line,

A: Expression of structural protein E2,

B: Expression of nonstructural proteins NS3 and NS4B,

10 C: Expression of NS5

FIG. 8 is a set of electrophoresis photographs showing the expression of the adenoviruses used in chimpanzee experiments of the present invention in 293A cell line,

15 A: Expression of structural protein E2,

B: Expression of nonstructural proteins NS3 and NS4B,

C: Expression of NS5

20

FIG. 9 is a set of graphs showing the cellular immune response 5 weeks after the first immunization. The induced cellular immune response varied in the mouse model according to the way to divide whole HCV gene to make a vaccine,

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FIG. 10 is a set of graphs showing the cellular immune response 3.4 weeks after the second immunization. The induced cellular immune response varied in the mouse model according to the way to divide whole HCV gene to make a vaccine,

FIG. 11 is a set of graphs showing the cellular immune response 5 weeks after the first immunization. The elimination of amino terminal 40 amino acids of HCV core enhanced cellular immune response in mouse model,

FIG. 12 is a set of graphs showing the cellular immune response 5 weeks after the first immunization or 3.4 weeks after the second immunization. Cellular immune response was better induced by HCV E2 DNA with transmembrane domain than by that without it,

FIG. 13 is a set of graphs in which mouse CD4+ T cells were separated by MACS (magnetic associated cell sorting) for the reaction with HCV E2 or core protein, and the resultant CD4+ T cells secreting IFN- γ or IL-4 were quantified,

Naive : Saline injected control,

G1 : pGX10 gDsΔ ST + pGX10-mIL-12 mutant
injection → pGX10 gDsΔ ST + pGX10-mIL-12 mutant
injection

G2 : pGX10 gDsΔ ST + pGX10-mIL-12 mutant
5 injection → rAd gDsΔ ST injection,

G3 : rAd gDsΔ ST injection → rAd gDsΔ ST
injection,

G4 : rAd gDsΔ ST injection → pGX10 gDsΔ ST +
pGX10-mIL-12 mutant injection

10.

FIG. 14 is a graph showing the result of the
cell lysis assay using CT26-hGHE2t cell line
expressing HCV E2 antigen,

- : control, ○ : G1, ◆ : G2,
15 ■ : G3, ▲ : G4

FIG. 15 is a schematic diagram showing the
vaccination regimen of the experiments of the
invention using chimpanzees with time to confirm the
20 effect of the vaccine of the invention,

FIG. 16 is a graph showing the result of IFN-γ
ELISPOT analysis of cellular immune response on the
second week after recombinant adenovirus boosting in
25 chimpanzee model,

FIG. 17 is a graph showing the amount of IFN- γ secreted by CD4+ T cells on the second week after boosting with a recombinant adenovirus in a chimpanzee model,

FIG. 18 is a graph showing the proliferation of CD4+ T cells on the second week after recombinant adenovirus boosting in chimpanzee model,

10

FIG. 19 is a graph showing HCV viral load in chimpanzees on the 0, 2nd and 4th week after the challenge with 100 CID₅₀ of infectious HCV-bk, which was performed on the 12th week after immunization with the vaccine,

15

FIG. 20 is a set of tables showing the amino acid sequences of HCV peptide pool used for the investigation of cellular immune response.

20

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

5

The reaction conditions for the molecular cloning process used in the examples of the present invention were as follow.

(1) Restriction enzyme treatment

10 All the enzymes were treated as follows. Plasmid DNA or 2 μg of purified PCR product (1 $\mu\text{g}/\mu\text{l}$) was treated with 20 unit of restriction enzyme (2 μl), and the buffer solution provided by the supplier (10 x solution) was added thereto. The distilled water
15 was then added to make the final volume 50 μl , which was reacted in a 37°C incubator for 2 hours.

(2) The ligation of DNA fragments and the transformation of *E. coli*

DNA solution was treated with restriction
20 enzyme and then electrophoresed on 0.8% agarose gel (GIBCO-BRL). The agarose gel containing a proper size DNA fragment was cut, and then the DNA was purified using gel extraction kit (QIAGEN). DNA fragments were ligated in the mixture of T4 DNA
25 ligase (Takara) and the buffer solution provided by

the supplier in a 16°C incubator for 10 hours. *E. coli* was transfected with the ligated DNA following the method of Sambrook, et al (Sambrook et al., *Molecular Cloning*, 2nd Ed. 1989).

5 (3) The confirmation of DNA having the ligated plasmids in the transfected *E. coli* and the purification thereof

DNA was purified from the transformed *E. coli* by the method of Sambrook et al., which was digested
10 with restriction enzyme based on the restriction enzyme map of the target DNA to confirm whether the target plasmids were correctly ligated. After the confirmation, pure DNA was mass-produced.

(4) PCR amplification

15 PCR was performed as follows. 200 pmol each of two oligoneucleotide primers, 20 ng of template DNA, 10 unit of Takara exTaq (polymerase), 5 µl of Takara exTaq 10x buffer solution, 5 µl of 2.5 mM dNTP mixture and distilled water were mixed to make the
20 final volume 50 µl. All the PCRs were performed as follows; predenaturation at 94°C for 4 minutes, denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, polymerization at 72°C for 1 minute per kb of a target PCR product (0.5 minute/0.5 kb, 3
25 minutes/3 kb), 30 cycles from denaturation to

polymerization, and final extension at 72°C for 5 minutes. GeneAmp PCR system 2400 (Perkin Elmer) was used for PCR. The obtained PCR products were electrophoresed on agarose gel, and then purified using the gel extraction kit (QIAGEN) by following the manufacturer's instruction. Then, the obtained products were digested with restriction enzyme as the above, which were used for further cloning process.

13 Other experiments concerning molecular cloning that were not explained herein were performed using the above method introduced by Sambrook et al with a slight modification.

15 Example 1: Construction of plasmids used in this invention

The present inventors constructed HCV plasmids (see FIG. 1 - FIG. 4) used in this invention as follows.

20

<1-1> Construction of pTV2 ST

pTV2 vector (Lee, J. Virol., 72, 8430, 1998; Cho, Vaccine, 17, 1136, 1999) was digested with PstI and XbaI to separate a 4.37 kb size vector. A 2.21

kb size insert was obtained by digesting the PCR product, which was amplified with the primers represented by SEQ. ID. No 1 and No 2 using Korean type HCV gene (see Korea patent No: 120928) that was isolated by the present inventors as a template, with the restriction enzymes PstI and XbaI, which was then combined with the above 4.37 kb size vector to construct pTV2 ST (FIG. 1).

10 <1-2> Construction of pTV2 Δ ST

pTV2 vector (Lee, *J. Virol.*, 72, 8430, 1998; Cho, *Vaccine*, 17, 1136, 1999) was digested with XbaI to separate a 4.78 kb size vector, and then treated with CIAP (Calf intestinal alkaline phosphatase) (Takara). A 2.07 kb size insert was obtained by digesting the PCR product, which was amplified with the primers represented by SEQ. ID. No 2 and No 3 using the pTV2 ST constructed in the above Example <1-2> as a template, with the restriction enzyme XbaI, which was then combined with the above 4.78 kb size vector to construct pTV2 Δ ST (FIG. 1).

<1-3> Construction of pTV2 gDsE2t

<1-3-1> Construction of pSK gDs

25 PCR was performed with the signal sequence of

glycoprotein D of Herpes simplex virus type 1 (gDs; Sisk, 1994, *J. Virol.*, 68: 766) using the primers represented by SEQ. ID. No 40 and No 41. pBluescript SK(+) vector (Stratagene) was digested
5 with EcoR V, into which the above PCR product was inserted, leading to the construction of pSK gDs (Lee, 1998 *J Virol* 72: 8430).

<1-3-2> Construction of pTZ HCV

10 In order to prepare a plasmid having a whole HCV base sequence, PCR was performed with the primers represented by SEQ. ID. No 42 and No 43 using Korean type HCV gene (see Korea Patent No: 120928) as a template. The obtained fragments were digested with
15 the restriction enzymes Hind III and Xba I, which were then inserted into pTZ vector (Sigma) digested with the same restriction enzymes to construct pTZ SN2. The prepared pTZ SN2 was digested with SphI, followed by digesting again with XbaI, resulting in a
20 5.8 kb size vector. pTV NS345 (Cho, 1999, *Vaccine*, 17:1136-44) was digested with XbaI, followed by the partial digesting again with SphI, leading to the separation of a 6.35 kb insert. The insert was combined with the above 5.8 kb vector to construct
25 pTZ HCV.

<1-3-3> Construction of pTV2 gDsE2t

pSK gDs constructed in the above Example <1-3-1> was digested with BglII and EcoRI to prepare a
5 vector. PCR was performed with the primers represented by SEQ. ID. No 4 and No 5 using pTZ HCV constructed in the above Example <1-3-2> as a template. The PCR product was digested with BglII and EcoRI, which was then combined with the vector
10 prepared above to construct pTV2 gDsE2t (Lee, J. Vol., 72: 8430) (FIG. 1).

<1-4> Construction of pTV2 gDsST

<1-4-1> Construction of pTV2 gDs

15 pTV2 gDsE2t constructed in the above Example <1-3> was digested with SpeI and EcoRV to separate a 3.62 kb size vector. A 1.28 kb size insert was obtained by PCR performed with the primers represented by SEQ. ID. No 44 and No 45 using pTV2
20 gDsE2t as a template, which was then combined with the above 3.62 kb size vector to construct pTV2 gDs.

<1-4-2> Construction of pTV2 gDsST

pTV2 gDs constructed in the above Example <1-4-1> was digested with AscI and XbaI to prepare a 4.86
25

kb vector. PCR was performed with the primers represented by SEQ. ID. No 6 and No 7 using pTV2 ST constructed in the above Example <1-1> as a template. The PCR product was digested with AscI and XbaI, which was combined with the vector obtained above, resulting in the construction of pTV2 gDsST (FIG. 1).

<1-5> Construction of pTV2 gDsE2

pTV2 gDsST constructed in the above Example <1-4> was digested with AscI and XbaI to prepare a 4.86 kb vector. PCR was performed with the primers represented by SEQ. ID. No 7 and No 8 using pTV2 ST constructed in the above Example <1-1> as a template. The PCR product was digested with AscI and XbaI, which was combined with the vector obtained above, resulting in the construction of pTV2 gDsE2 (FIG. 1).

<1-6> Construction of pTV2 gDsΔ SN2

pTV2 gDs constructed in the above Example <1-4-1> was digested with AscI and XbaI to prepare a 4.89 kb vector. PCR was performed with the primers represented by SEQ. ID. No 9 and No 10 using pTZ HCV constructed in the above Example <1-3-2> as a template. The PCR product was digested with AscI and XbaI, which was combined with the vector obtained

above, resulting in the construction of pTV2 gDsΔ SN2 (FIG. 1).

<1-7> Construction of pTV2 SN2

5 pTV2 gDsΔ SN2 constructed in the above Example
<1-6> was digested with PstI and NotI to prepare a
5.98 kb vector. pTV2 ST constructed in the above
Example <1-1> was digested with PstI and NotI to
separate a 1.67 kb size insert. The 1.67 kb size
10 insert was then combined with the vector obtained
above, resulting in the construction of pTV2 SN2 (FIG.
1).

<1-8> Construction of pTV2 Δ SN2

15 pTV2 vector (Lee, *J. Virol.*, 72, 8430, 1998;
Cho, *Vaccine*, 17, 1136, 1999) was digested with PstI
and XbaI to separate a 4.73 kb size vector. A 2.80
kb size insert was obtained by digesting the PCR
product, which was amplified with the primers
20 represented by SEQ. ID. No 46 and No 47 using the pTZ
HCV constructed in the above Example <1-3-2> as a
template, with the restriction enzymes PstI and XbaI,
which was then combined with the above 4.73 kb size
vector to construct pTV2 Δ SN2 (FIG. 1).

25

<1-9> Construction of pTV2 SN5

pTV2 ST constructed in the above Example <1-1> was digested with NotI and XbaI to separate a 6.25 kb size vector. pTZ HCV constructed in the above Example <1-3-2> was digested with NotI and XbaI to separate a 7.50 kb size insert. The 7.50 kb size insert was then combined with the vector obtained above, resulting in the construction of pTV2 SN5 (FIG. 1).

10

<1-10> Construction of pTV2 gDsSTt

pTV2 vector (Lee, *J. Virol.*, 72, 8430, 1998; Cho, *Vaccine*, 17, 1136, 1999) was digested with PstI and XbaI to prepare a 4.73 kb size vector. A 2.40 kb size insert was obtained by digesting the PCR product, which was amplified with the primers represented by SEQ. ID. No 11 and No 12 using the pTV2 gDsST constructed in the above Example <1-4> as a template, with the restriction enzymes PstI and XbaI, which was then combined with the above 4.73 kb size vector to construct pTV2 gDsSTt (FIG. 1).

15

20

<1-11> Construction of pTV2 gDsΔ ST

pTV2 gDs constructed in the above Example <1-4-1> was digested with AscI and XbaI to prepare a 4.89

25

kb size vector. A 2.07 kb size insert was obtained by digesting the PCR product, which was amplified with the primers represented by SEQ. ID. No 9 and No 7 using the pTV2 ST constructed in the above Example <1-1> as a template, with the restriction enzymes AscI and XbaI, which was then combined with the above 4.89 kb size vector to construct pTV2 gDsΔ ST (FIG. 1).

10 <1-12> Construction of pTV2 gDsΔ STt

pTV2 vector (Lee, *J. Virol.*, 72, 8430, 1998; Cho, *Vaccine*, 17, 1136, 1999) was digested with PstI and XbaI to prepare a 4.73 kb size vector. A 2.28 kb size insert was obtained by digesting the PCR product, which was amplified with the primers represented by SEQ. ID. No 11 and No 12 using the pTV2 gDsΔ ST constructed in the above Example <1-11> as a template, with the restriction enzymes PstI and XbaI, which was then combined with the above 4.73 kb size vector to construct pTV2 gDsΔ STt (FIG. 1).

<1-13> Construction of pGX10 gDsΔ ST

<1-13-1> Construction of pGX10

<1-13-1-1> Construction of pTV-3

25 2 μg of vector 'pMT-2' (Sambrook, *Molecular*

cloning, 2nd Ed., Vol. 3, 16.20; Kaufman RJ, *Mol. Cell Biol.* 9,946-958, 1989) was digested with HpaI (20 unit) and NheI (20 unit) by the same method as explained above, and Klenow fragment (New England Biolabs) (5 unit) and dNTP (Takara) were added to make the final concentration 100 μ M, which was treated at 25°C for 30 minutes to make blunt end. After completing electrophoresis on agarose gel, the obtained 0.7 kb fragment (the whole adenovirus VAI (Viral Antagonist I) and a part of SV40 polyA were included) was inserted in the specific HpaI site of SV40 polyA region of the vector pTV-2 (Lee, 1998 *J Virol.*, 72,8430-36), resulting in the construction of 5.3 kb size vector 'pTV-3'.

15

<1-13-1-2> Construction of pGX-1

PCR was performed using the vector pTV-3 constructed in the above Example <1-13-1-1> as a template with the primers represented by SEQ. ID. No 13 and No 14, and the PCR product (2.0 kb) was digested with NruI. PCR was also performed using the vector pZero-2 (Invitrogen) as a template with the primers represented by SEQ. ID. No 15 and No 16, and the product was digested with SspI. The obtained fragment (1.8 kb) was ligated with the above DNA

25

fragment (2.0 kb) to construct 3.8 kb size vector 'pGX-1'.

<1-13-1-3> Construction of pGX10

5 The vector pGX-1 constructed in the above Example <1-13-1-2> was digested with restriction enzymes XbaI and SalI, and the bigger DNA (3.1 kb) of the two fragments was separated by the method used for the ligation of DNA fragments and for the transformation of *E. coli*. The vector pGL3-enhancer (Promega) was also digested with the restriction enzymes XbaI and SalI, and the smaller DNA (0.5 kb) of the obtained two fragments was separated as the above. The two separated fragments were ligated to construct the vector pGX10 (FIG. 2).

<1-13-2> Construction of pGX10 gDsΔ ST

20 A 3.4 kb size vector was obtained by digesting pGX10, constructed in the above Example <1-13-1>, with PstI and XbaI, which was then ligated to a 2.22 kb size insert obtained by digesting pTV2 gDsΔ ST which was constructed in the above Example <1-11> with PstI and XbaI to construct pGX10 gDsΔ ST containing the base sequence (gDsΔ ST) represented by SEQ. ID. No 50 (FIG. 2). The constructed plasmid was

deposited at Korean Culture Center of Microorganisms
on August 29, 2002 (Accession No: KCCM 10415).

<1-14> Construction of pGX10 NS34

5 A 3.56 kb size vector was obtained by digesting
pGX10 gDsΔ ST constructed in the above Example <1-13>
with AscI and XbaI. PCR was performed using pTZ HCV,
constructed in the above Example <1-3-2>, with the
primers represented by SEQ. ID. No 17 and No 18. The
10 above vector was ligated to a 2.82 kb insert prepared
by digesting the PCR product with PstI and XbaI,
resulting in the construction of pGX10 NS34
containing the base sequence (NS34) represented by
SEQ. ID. No 51 (FIG. 2). The obtained plasmid was
15 deposited at Korean Culture Center of Microorganisms
on August 29, 2002 (Accession No: KCCM 10417).

<1-15> Construction of pGX10 NS5

20 A 3.4 kb size vector was constructed by
digesting pGX10, constructed in the above Example <1-
13-1>, with Asp718 and XbaI. PCR was performed using
pTZ HCV, constructed in the above Example <1-3-2>, as
a template with the primers represented by SEQ. ID.
No 19 and No 20. A 3.12 kb size insert, obtained by
25 digesting the PCR product with Asp718 and XbaI, was

ligated to the above vector, resulting in the construction of pGX10 NS5 containing the base sequence (NS5) represented by SEQ. ID. No 52 (FIG. 2). The obtained plasmid was deposited at Korean Culture
5 Center of Microorganisms on August 29, 2002 (Accession No: KCCM 10416).

<1-16> Construction of pGX10 hIL-12m (pGX10-hp35/IRES/hp40-N222L)

10 <1-16-1> Construction of pSK-hp35 and pSK-hp40

After cloning cDNAs of 820 bp size human p35 subunit and 1050 bp size p40 subunit from NC37 cells (American Type Culture Collection; ATCC), human B cells activated by PMA (phorbol myristic acetate),
15 using RT-PCR (Reverse transcriptase-polymerase chain reaction, PCR System 2400, Perkin Elmer), PCR was performed with the primers represented by SEQ. ID. No 21 and No 22 for the 820 bp human p35 subunit, and with the primers represented by SEQ. ID. No 23 and No
20 24 for the 1050 bp size p40 subunit.

The amplified cDNAs were subcloned into the starting vector pBluescript SK+ (Stratagene). Each gene of p35 and p40 subunit was also inserted into the SamI site of the vector to construct pSK-hp35
25 (3.8kb) and pSK-hp40 (4.0kb).

<1-16-2> Construction of pSK-IRES

In order to construct a vector (bicistronic vector) expressing genes encoding p35 and p40 subunits, RT-PCR was performed using the primers represented by SEQ. ID. No 25 and No 26, to obtain IRES (Internal Ribosome Entry Site) gene of EMCV (Encephalomyocarditis virus). The IRES gene was digested with EcoRV, which was then inserted into
10 EcoRV site of pBluescript SK+ to construct the vector pSK-IRES (3.5 kb).

<1-16-3> Construction of pSK-hp35/IRES

A 3.5 kb size vector was obtained by digesting
15 pSK-IRES, constructed in the above Example <1-16-2>, with the restriction enzyme EcoRV. pSK-hp35 constructed in the above Example <1-16-1> was also digested with the restriction enzymes EcoRV and NotI. Then, hp35 fragment (0.8 kb) which was filled in with
20 T4 DNA polymerase was inserted into the above vector to construct pSK-hp35/IRES (4.3 kb).

<1-16-4> Construction of pSK-hp35/IRES/hp40

The hp40 fragment (1.0 kb), obtained by
25 digesting the vector pSK-hp40 constructed in the

above Example <1-16-1> with NcoI and NotI, was inserted into the digested area of pSK-hp35/IRES constructed in the above Example <1-16-3> by the same restriction enzymes. As a result, a vector that can express both genes encoding p35 subunit and p40 subunit simultaneously was constructed. And the vector was digested with the restriction enzymes SmaI and ClaI, followed by re-ligation using T4-ligase, so that a part of the restriction enzyme site in front of hp35 was eliminated. At last, the vector pSK-hp35/IRES/hp40 was constructed.

<1-16-5> Construction of pSK-hp40-N222L

In order to replace aspartic acid, the 222nd amino acid of hp40, by leucine, PCR was performed with the primers represented by SEQ. ID. No 27 and No 28 using pKS-hp40 constructed in the above Example <1-16-1> as a template. Likewise, secondary PCR was performed using the primers represented by SEQ. ID. No 29 and No 30. So, two PCR fragments, which were sharing the common site including a mutational point, were made. The secondary PCR was performed using the mixture of the above fragments as a template and the flanking primers. As a result, a fusion product was obtained, which was inserted into the vector

pBluescriptSK+ (Stratagene, 3.0kb) prepared by digesting the fragments with SmaI to construct the plasmid pSK-hp40-N222L (4.0 kb).

5 <1-16-6> Construction of pSK-hp35/IRES/hp40-N222L

hp40-N222L fragment of pSK-hp40-N222L constructed in the above Example <1-16-5> was substituted with hp40 fragment of pSK-hp35/IRES/hp40 constructed in the above Example <1-16-4> using the
10 restriction enzymes NcoI and NotI to construct the plasmid pSK-hp35/IRES/hp40-N222L (5.3 Kb).

<1-16-7> Construction of pGX10-hp35/IRES/hp40-N222L

In order to transfer the fragment
15 hp35/IRES/hp40-N222L to a vector that is able to express the gene in mammalian cell, PCR was performed using pSK-hp35/IRES/hp40-N222L constructed in the above Example <1-16-6> as a template and the primers represented by SEQ. ID. No 31 and No 32, so that the
20 vector was amplified to produce the fragment hp35/IRES/hp40-N222L. The obtained fragment was digested with the restriction enzyme XhoI, which was then inserted into XhoI site of pGX10 prepared in the above Example <1-13-1>. As a result, the 5.9 kb size
25 plasmid 'pGX10-hp35/IRES/hp40-N222L' (pGX10 hIL-12m)

containing the base sequence represented by SEQ. ID.
No 53 (hIL-12m) was produced (FIG. 3).

Example 2: Construction of the recombinant adenovirus

5 used in this invention

<2-1> Construction of pShuttleCMV gDsΔ ST

<2-1-1> Construction of pShuttleCMV gDsΔ ST:H77C

pShuttleCMV (Q biogene Co.) was digested with
BglII and XbaI to separate a 7.5 kb size vector. A
10 2.07 kb size insert was obtained by digesting the PCR
product, which was amplified with the primers
represented by SEQ. ID. No 33 and No 34 using pCV-
H77C (Masayuki, 1998 Virology 244: 161-72) as a
template, with the restriction enzymes BglII and XbaI,
15 which was then combined with the above 7.5 kb size
vector to construct pShuttleCMV gDsΔ ST:H77C.

<2-1-2> Construction of pShuttleCMV gDsΔ ST

pShuttleCMV gDsΔ ST:H77C constructed in the
20 above Example <2-1-1> was digested with AscI and XbaI
to separate a 7.43 kb size vector. pGX10 gDsΔ ST
constructed in the above Example <1-13-2> was also
digested with AscI and XbaI to obtain a 2.07 kb size
insert. The obtained insert was ligated with the

above 7.43 kb size vector to construct pShuttleCMV
gDsΔ ST.

<2-2> Construction of pShuttleCMV gDsNS34

5 <2-2-1> Construction of pGX10 gDsNS34

pGX10 gDsΔ ST constructed in the above Example
<1-13-2> was digested with AscI and XbaI to separate
a 3.5 kb size vector. A 2.81 kb size insert was
obtained by digesting the PCR product, which was
10 amplified with the primers represented by SEQ. ID. No
48 and No 49 using pTZ HCV constructed in the above
Example <1-3-2> as a template, with the restriction
enzymes AscI and XbaI, which was then combined with
the above 3.5 kb size vector to construct pGX10
15 gDsNS34.

<2-2-2> Construction of pShuttleCMV gDsNS34

pShuttleCMV gDsΔ ST constructed in the above
Example <2-1-2> was digested with AscI and XbaI to
20 separate a 7.43 kb size vector. pGX10 gDsNS34
constructed in the above Example <2-2-1> was also
digested with AscI and XbaI to obtain a 2.81 kb size
insert. The obtained insert was ligated with the
above 7.43 kb size vector to construct pShuttleCMV
25 gDsNS34.

<2-3> Construction of pShuttleCMV NS5

pShuttleCMV (Q biogene Co.) was digested with Asp718 and XbaI to separate a 7.46 kb size vector. A
5 3.12 kb size insert was obtained by digesting the PCR product, which was amplified with the primers represented by SEQ. ID. No 19 and No 20 using pTZ HCV constructed in the above Example <1-3-2> as a template, with the restriction enzymes Asp718 and
10 XbaI, which was then combined with the above 7.46 kb size vector to construct pShuttleCMV NS5.

<2-4> Construction of pGX10 mL-12m

<2-4-1> Construction of wild-type mouse IL-12
15 expression vector

<2-4-1-1> Construction of pSK-mp35/IRES/mp40

In order to prepare a vector simultaneously expressing the genes each coding mouse p40 subunit and p35 subunit, the vector pSK-IRES, constructed in
20 the above Example <1-16-2>, containing IRES of EMCV was digested with the restriction enzymes NcoI and BamHI. Mouse IL-12p40 PCR product (Schoenhaut, J. Immunol., 1999, 148:3433-3440) was also digested with the same restriction enzymes, resulting in a p40 DNA
25 fragment. The p40 DNA fragment was inserted into the

above vector to construct pSK-IRES/mp40. Mouse IL-12p35 product (Schoenhaut, J. *Immunol.*, 1992, 148:3433-3440) was digested with BamHI, and the end of the fragment was filled in by T4 DNA polymerase. The fragment was inserted into pSK-IRES/mp40 treated with ClaI and T4 DNA polymerase. As a result, the plasmid pSK-mp35/IRES/mp40 was constructed, in which p35, IRES and p40 were arranged in that order.

10 <2-4-1-2> Construction of the expression vector pCIN-mp35/IRES/mp40

mp35/IRES/mp40 constructed by the same method as used in the above Example <2-4-1-1> was inserted into XhoI and NotI sites of pCI-neo vector (Promega Co.), to construct the expression vector 'pCIN-mp35/IRES/mp40' that was able to express active form of IL-12p70 in mammalian cells.

20 <2-4-2> Construction of the expression vector pCIN-mp40

In order to construct a plasmid expressing wild-type mouse p40 subunit, the vector pSK-mp35/IRES/mp40 constructed in the above Example <2-4-1-1> was digested with the restriction enzymes NcoI and SacI, to obtain p40 fragment, which was, then,

inserted into the vector pGEX-KG (Clontech Co., USA) treated with the same restriction enzymes. The prepared pGEX-KG-mp40 was treated with EcoRI and NotI, which was inserted into the EcoRI and NotI sites of pCI-neo. As a result, the expression vector pCIN-mp40 was obtained.

<2-4-3> Construction of the expression vector pCIN-mp35

In order to construct a plasmid expressing wild-type p35 subunit, the vector pSK-mp35/IRES/mp40 constructed in the above Example <2-4-1-1> was digested with the restriction enzymes XhoI and EcoRI, to obtain p35 fragment, which was, then, inserted into the XhoI and EcoRI sites of the vector pCI-neo treated with the same restriction enzymes to construct the expression vector pCIN-mp35.

<2-4-4> Construction of the expression vector pCIN-mp40-N220L

In order to replace aspartic acid, the 220th amino acid of mp40, by leucine, PCR was performed using pCIN-mp40 constructed in the above Example <2-4-2> as a template and primers represented by SEQ. ID. No 35 and No 36. At that time, the restriction

enzyme SacI recognition site was included for easy discrimination. Thus, the vector pCIN-mp40-N220L, which contained mouse IL-12p40 mutant gene and was able to be expressed in animal cells, was prepared.

5

<2-4-5> Construction of pTV2-mp35/IRES/mp40-N220L

In order to construct a DNA expressing the genes coding p40 and p35 subunits simultaneously for DNA immunization, pTV2 vector (Lee, J. Virol., 72:8430-8436, 1998; Cho, Vaccine, 17:1136-1144, 1999), which is an eukaryotic expression vector and has been used as a DNA vaccine vector in small animal models, was digested with the restriction enzymes Asp718 and NotI. mp35/IRES/mp40 fragment was prepared by digesting pSK-mp35/IRES/mp40, constructed in the above Example <2-4-1-1>, with the same restriction enzymes, which was inserted into the above restriction enzyme sites to construct the vector pTV2-mp35/IRES/mp40. And in order to construct a vector containing Asn-220 mutant gene of mouse IL-12p40 and expressing p35 at the same time, pSK-mp35/IRES/mp40 was digested with NcoI and NotI, into which mp40-N220L fragment obtained by cutting pCIN-mp40-N220L by the same enzymes was inserted, resulting in the construction of pSK-mp35/IRES/mp40-

25

N220L. pTV2-mp35/IRES/mp40 was digested with EcoRV and NotI to eliminate mp40, into which mp40-N220L fragment obtained by digesting pSK-mp35/IRES/mp40-N220L with the same enzymes was inserted, resulting
5 in the construction of the vector pTV2-mp35/IRES/mp40-N220L. The vector was deposited at Gene Bank of Korea Research Institute of Bioscience and Biotechnology on February 29, 2000 (Accession No: KCTC 0745BP).

10

<2-4-6> Construction of pGX10 mL-12

The DNA fragment (2.5kb), obtained by digesting the vector pTV2-mp35/IRES/mp40-N220L with SacII and NotI, was combined with the other DNA fragment
15 (3.4kb), obtained by digesting pGX10 with the same restriction enzymes, to construct the vector pGX10 mL-12 mutant (5.9kb).

<2-5> Construction of the recombinant adenovirus rAd-gDsΔ ST 20

The recombinant adenovirus rAd-gDsΔ ST of the present invention was prepared by using pAdEasy (Trade mark) vector system (Q. Biogene Co.). pShuttleCMV gDsΔ ST, constructed in the above Example
25 <2-1>, was digested with PmeI, which was used, along

with the vector pAdEasy, for transformation of *E. coli* strain BJ5183 by electroporation. rAd gDsΔ ST containing the base sequence represented by SEQ. ID. No 50 (gDsΔ ST) was constructed by the homologous recombination process within the bacterial strain (FIG. 4). DNA was extracted from the transformed cells and then digested with the restriction enzyme PacI. 293A cells (ATCC) which had been cultured on 60 mm dish were transfected with the DNA by calcium phosphate method. 10 days later, the transfected cells were frozen and then melted, which was repeated three times. Supernatant was separated and used for re-transfection of freshly cultured 293A cells on 100mm dish for amplification. The virus was cultured for three days with the same procedure, and at last, amplified on 150 mm dish until the quantity of 293A cells were increased to 30 units of 150 mm dishes. 293A cells were harvested, from which only the pure recombinant adenoviruses were purified according to the manufacturer's instruction (Q. Biogene Co.). The purified viruses were quantified by the method of TCID₅₀ (Tissue Culture Infectious Dose 50). As a result, about 1×10^{11} pfu of recombinant adenovirus rAd gDsΔ ST were obtained. The above recombinant adenovirus was deposited at Korean Culture Center of

Microorganisms on August 29, 2002 (Accession No: KCCM 10418).

5 <2-6> Construction of the recombinant adenovirus rAd-gDsNS34

rAd-gDsNS34 containing the base sequence represented by SEQ. ID. No 54 (gDsNS34) was constructed using pShuttleCMV gDsNS34 constructed in the above Example <2-2-2> by the same method as used for the production of rAd-gDs Δ ST in the above Example <2-5>. The recombinant adenovirus rAd-gDsNS34 was deposited at Korean Culture Center of Microorganisms on August 29, 2002 (Accession No: KCCM 10420).

15

<2-7> Construction of the recombinant adenovirus rAd-NS5

rAd-NS5 containing the base sequence represented by SEQ. ID. No 52 (gDsNS34) was constructed using pShuttleCMV NS5 constructed in the above Example <2-2-2> by the same method as used for the production of rAd-gDs Δ ST in the above Example <2-5> (FIG. 4). The recombinant adenovirus rAd-NS5 was deposited at Korean Culture Center of Microorganisms on August 29, 2002 (Accession No: KCCM

25

10419).

Example 3: Identification of the expressions of core,
E2, NS3, NS4 and NS5 in HCV DNA vaccine and
recombinant adenovirus vaccine (FIG. 5 - FIG. 8)

The present inventors confirmed the expressions of HCV antigens - core, E2, NS3, NS4 and NS5 - in various DNA vaccine plasmids and recombinant adenovirus vaccines constructed in the present invention using the methods explained above. At first, COS-7 cells were used to confirm the expressions of the plasmids constructed in this invention. COS-7 cells were cultured in DMEM medium (Gibco BRL Co.) containing 10% FBS (fetal bovine serum). 5×10^5 cells were plated onto 60 mm dish for the culture. Next day, the cells were transfected with 10 μ g each of the plasmids presented in the FIGs. 5, 6 and 7, using calcium phosphate method. 36 hours later, cells were collected for electrophoresis. In order to normalize the variation of transfection efficiency, 5 μ g of luciferase gene was mixed with the indicated plasmids before the transfection, and cell lysate having the same activity level of

luciferase was taken for electrophoresis. 293A cells were used to confirm the expression of the recombinant adenovirus. 293A cells which had been cultured on 60 mm dish were infected with the recombinant adenovirus by 1×10^6 pfu of the recombinant virus per 5×10^5 cells, as seen in FIG. 8, and 36 hours later, the cells were harvested for electrophoresis. After running the electrophoresis on 10% SDS-polyacrylamide gel, Western blotting was performed. In order to detect E2 protein, anti-E2 monoclonal antibody (Lee, 1999 *J Virol* 73: 11-8) was used. In order to detect Δ core protein in which N-terminal 40 amino acids were removed, anti-core polyclonal antibody was used. And in order to detect non-structural protein such as NS3, NS4 and NS5, serum of HCV patient was used.

FIG. 5 shows the expression of E2 protein in the plasmids used in small animal experiments. In that figure, (A) provides a good comparison of expression levels of the plasmids used in the experiments, and (B) shows the difference of molecular weights after the elimination of transmembrane domain. E2 has 38 KDa in molecular weight, but a band of 65-70 KDa was detected because

of glycosylation in endoplasmic reticulum (ER) and golgi complex. In general, the longer the insert introduced in a plasmid was, the lower the expression level was. pTV2 SN5 formed a specific band at the right position, although it was faint but good enough to confirm the expression. The changes in migration rate of E2 after the elimination of transmembrane domain suggested that the domain was correctly removed.

FIG. 6 shows the expression of core and its elimination of amino terminal 40 amino acids. Core has 21 KDa in molecular weight. It increased to 24 KDa when gDs was inserted, and decreased to 4.5 KDa after amino terminal 40 amino acids were removed. In order to track the position of core on electrophoresis, pTV2 core tested by the present inventors was used. As shown in the figure, the antibody to core specific expression product was seen as a clear band, suggesting that core was expressed and amino terminal 40 amino acids were correctly eliminated. When pTV2 gDs Δ ST was used, the core band seemed to be unclear, comparing to the cases using other plasmids, which, though, did not suggest that the expression of core was weak. First, the major B cell epitope inducing antibody response

existed at amino terminus of core, according to previous reports (Sallberg, 1992 *Immunol Lett* 33:27; Kakimi, 1995 *J Gen Virol* 76: 1205; Harase, 1995 *Immunol Cell Biol* 73:346). Thus, even though the expression levels of core in the plasmids were comparable, the antibody is not likely to bind to the core protein in which amino terminus was removed. So, the band was seen lighter than that of a wild type. Second, core, E1 and E2 proteins are processed by the host protease into an individual gene product in that order after polyprotein was produced in the host cell transfected with the plasmids 'pTV2-ST', '-gDsST', '-gDsΔ ST', etc. Based on that, the expression level of each protein was believed to be similar during the synthesis. As seen in FIG. 5A, the level of E2 expression in pTV2 gDsST was similar to that in pTV2 gDsΔ ST. Thus, the weak core band observed in pTV2 gDsΔ ST was not because core was expressed less but because there was a limitation in the binding affinity of anti-core antibody to core protein without amino terminus.

FIG. 7 shows the expression of the plasmid used in the experiments with chimpanzees. E2 antibody specific band was observed at the correct position in pGX10 gDsΔ ST (A), and 67 KDa NS3 protein and 27 KDa

NS4B protein were confirmed in pGX10 NS34 (B). Generally, when whole non-structural is expressed NS5 protein is detected as divided forms into 49 KDa NS5A and 66 KDa NS5B that are cleavage product by NS3 protease of hepatitis C virus. But in transfected cells with pGX10 NS5 alone, the 115 KDa single band was detected since protease was not provided in this case (C). A specific 100 KDa band was observed, which seemed to be a cleavage product generated by unknown processing during the expression in the cells.

FIG. 8 shows the expression of a recombinant adenovirus. The equal explanation to the case seen in FIG. 7 can be applied to rAd gDs Δ ST (A) and rAd NS5 (c). The molecular weight of rAd gDsNS34 (B) increased to 71 KDa by substituting 67 KDa NS3 protein with gDs, and additionally by the glycosylation occurring after changing the location to ER by inserting gDs.

All the plasmids used in the present invention were confirmed by nucleotide sequencing for translation start site and joining regions in clong procedure. For the identification of structural protein expressions, E2 expression was examined and core expression was tested in some of those proteins. E1 expression was also investigated, but the

corresponding band was hardly detected because of low sensitivity and inaccuracy of the antibody used. Nevertheless, E1 was believed to be expressed correctly, considering the fact that core and E2
5 expressions were clearly observed during the expression process having the order of core-E1-E2.

Through the preferred embodiments of the invention, the present inventors suggest that the
10 target gene product can be expressed after administration *in vivo*, by observing the expressions of the plasmid and the recombinant adenovirus of the invention in cultured animal cells.

15 Example 4: Immunization of mice with a HCV DNA vaccine and an adenovirus vaccine

The mentioned plasmid was dissolved in 100 μl of PBS, which was used as a DNA vaccine for the immunization of small animals. The first
20 administration was performed by injecting 100 μl of a DNA vaccine into both legs (50 μl per each hind leg muscle) of 6 mice per each group. In some cases, the secondary intramuscular injection followed 4 weeks later. In the example (FIG. 9 - FIG. 12) aiming at

enhancing the immunogenicity by antigen engineering, 100 μ g of DNA was used. Otherwise, 50 μ g of DNA prepared by mixing 40 μ g of the plasmid pGX10 gDs Δ ST and 10 μ g of the mutant pGX10 mIL-12, was used for the immunization in FIG. 13 and FIG. 14. 5×10^7 pfu of rAd gDs Δ ST was dissolved in saline, which was used as a recombinant adenovirus vaccine (rAd). In order to investigate cellular immune response induced after immunization, spleen cells were isolated at the indicated time. A control group was injected with saline alone.

Example 5: IFN- γ ELISPOT analysis with mice

IFN- γ ELISPOT analysis was performed according to the manufacturer's instruction using IFN- γ ELISPOT kit (Cat# M34201-H, MABTECH Co.) after pooling spleen cells taken from 2-3 immunized mice. Particularly, the coating antibody (1-D1K) for IFN- γ was diluted with PBS by 5 μ g/ml, and 50 μ l of the diluted antibody solution was distributed into a 96 well plate (Millipore, 0.45m, Cat# MAHAS4510, Bedford, MA), which was then left at room temperature for over 12 hours. The remaining antibody solution was removed by suction. The plate was washed with PBS

twice, and 200 μ l of medium for animal cell culture (RPMI-1640 containing 50 units/ml penicillin, 50 μ g/ml streptomycin, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 20 units/ml recombinant mIL-2 and 10% FBS (standard fetal bovine serum, HyClone, Lot. #AJH10775)) was added into each well. After leaving the plate at 37°C for over 2 hours, the remaining solution was removed by suction again. The isolated spleen cells were added into each well by 1×10^6 , 3.3×10^5 and 1.1×10^5 cells/well respectively. In order to investigate the HCV antigen specific immune response caused by CD8+ T cells, the cell line 'CT26-hghE2t', which has been known to express H-2d MHC class I and E2 protein of hepatitis C virus stably at the same time (Song, J. Virol., 74:2920-2925, 2000), was added into each well by 2×10^4 cells/well. Then, the 96 well plate was cultured in a 37°C, 5% CO₂ incubator without agitation for 20 hours. After stimulation, contents in the 96 well plate were poured out, and washed with washing solution (PBS-T, 0.1%) containing 0.1% tween 20 (Sigma, Cat# D8654) four times. Biotin-labeled mAb (7-B6-1 biotin) was diluted with blocking buffer, prepared by adding 1% BSA to the washing solution, to adjust the concentration to 1 μ g/ml. That was added

into each well by 50 μ l, followed by incubation for 2 hours. After being washed with the washing solution (PBS-T, 0.1%) four times, the plate was filled with streptavidin-HRP solution diluted by the blocking buffer (1:100), which was then reacted for 1 hour. On completion of the reaction, AEC substrate solution was used to induce color development. The color reaction was stopped using tap water when a required size spot was observed (5-10 minutes). The 96 well plate was dried at room temperature, and then, the number of cells secreting IFN- γ was measured by using a microscope.

For the ELISPOT analysis of the invention, spleen cells were used either immediately after being taken (direct ELISPOT) or after being expanded for 5 days stimulation with CT26-hgHE2t cells at the mixing volume of spleen cells to CT26-hgHE2t cells: 1.5×10^7 to 1×10^6 (expanded ELISPOT).

Example 6: Immune response of HCV E2 specific cytotoxic T lymphocyte (CTL)

The immune response of cytotoxic T lymphocyte was investigated using spleen cells taken from 2-3

mice of each group after treating those mice with a DNA vaccine and a recombinant adenovirus vaccine. In order to stimulate HCV E2 specific cytotoxic T lymphocyte, CT26-hgHE2t cell line (H-2d restricted),
5 in which E2 protein is expressed, was treated with mitomycin-C for 30 minutes for the following reaction. Spleen cells were isolated from mice of each group, and about 1.5×10^7 spleen cells were mixed with 10^6 CT26-hgHE2t cells in cell culture media (RPMI-1640
10 medium + 10% FBS + 2 mM glutamine + 20 μ M β -mercaptoethanol + 20 U/ml of mIL-2), followed by stimulation in a 37°C CO₂ incubator for 5 days. New CT26-hgHE2t cells, target cells in this embodiment, were plated into each well by 10^4 cells. after
15 treatment with 1 μ Ci of ⁵¹Cr for 2 hours and washing with cell culture media three times. Spleen cells expanded for 5 days were used as effector cells for CTL assay. The effector cells and the target cells were mixed at three different ratios indicated in the
20 figure, which was then reacted for 5 hours. Supernatant was obtained from the culture solution, and the activity of the cytotoxic T lymphocytes was examined by measuring the amount of released ⁵¹Cr with a gamma radioactivity counter. At that time, a
25 negative control group was prepared by adding only

medium to investigate the spontaneous release of ^{51}Cr .
A positive control group was also prepared by adding
2% tween solution to induce maximum release of ^{51}Cr
in every target cells. The way to measure the
5 activity of cytotoxic T lymphocyte was as follows.

$$\% \text{ Specific Lysis} = (R_{\text{Max}} - R_{\text{eff}}) / (R_{\text{Max}} - R_{\text{spon}}) \times 100$$

R_{Max} : Maximum Release
10 R_{eff} : Effector Release
 R_{spon} : Spontaneous Release

Example 7: Enhancement of immunogenicity by antigen
engineering of a DNA vaccine-1

15 (FIG. 9, FIG. 10: The effect of the insert size in an
expression vector on the induction of cellular immune
response)

The present inventors investigated what size of
insert in a DNA vector was best for the induction of
20 HCV-specific cellular immune response.

For the comparison, a plasmid expressing E2
only and other plasmids having a longer size by
including other areas in addition to E2 were used.
E2 is translated after E1. At this time,

transmembrane domain of E1 functions as a signal sequence for translocation of E2 to ER (Endoplasmic Reticulum). Thus, as for the plasmid expressing E2 only, HSV gDs was inserted at amino terminus of E2 in order to locate the expressed protein to ER. Taking the plasmid as a control vector, other plasmids with different insert lengths, classified in three groups, were compared. First, as a category of plasmids expressing intact HCV core in cytosol, pTV2-ST expressing from core to E2, pTV2-SN2 expressing from core to NS2, and pTV2-SN5 expressing whole HCV gene from core to NS5 were used for the comparison. Second, as a category of plasmids expressing Δ core in cytosol which is deficient of amino terminal 40 amino acids, pTV2- Δ ST and pTV2- Δ SN2 were used. And third, as a category of plasmids expressing Δ Core in ER, pTV2-gDs Δ ST and pTV2-gDs Δ SN2 were used. All the above plasmids were used for immunization by 100 μ g each. 5 weeks after the immunization and 3.4 weeks after the second immunization, the response of CD8+ T cells was investigated. CT26-hghE2t cell line was used as a stimulant to investigate E2-specific cytotoxic T lymphocyte response or IFN- γ ELISPOT analysis with E2 since the cell line did not express MHC class II but expressed MHC class I, suggesting

that the cell line stimulated CD8+ T cells selectively. That approach was proved to be consistent through the evaluation of cytotoxic T lymphocyte response and IFN- γ ELISPOT analysis performed after stimulating CT26-hgHE2t cell lines for 5 days, showing the similar result between the two analyses.

Cellular immune response was observed 5 weeks after the first immunization. As a result, when the first category of plasmids were used, the IFN- γ ELISPOT response observed therein was 47% with pTV2 ST, 23% with pTV2 SN2 and 3% with pTV2 SN5 by taking the rate with pTV2 gDsE2 as a standard ($p < 0.0001$), and 84%, 50%, 18% and 2% of CTL response was observed respectively (Fig. 9). So, cellular immune response was decreased in proportion to the insert length in a DNA vaccine. On the other hand, in the case of plasmid in which Δ Core was expressed in cytoplasm or ER, pTV2 Δ ST showed 77% response, pTV2 Δ SN2 did 31%, pTV2 gDs Δ ST did 84%, and pTV2 gDs Δ SN2 showed 20% of IFN- γ ELISPOT responses, compared with pTV2 gDsE2, suggesting that the plasmid expressing from core to E2 showed similar cellular immune response to the plasmid expressing E2 alone, but the response dropped

rapidly after using the plasmid containing more than NS2. The similar result was observed in cytotoxic T lymphocyte response, in which pTV2 gDsE2 showed 84% response, pTV2 Δ ST did 79%, pTV2 Δ SN2 showed 54%,
5 and further pTV2 gDs Δ ST showed 68%, pTV2 gDs Δ SN2 did 53% respectively. Cellular immune response was investigated again 3.4 weeks after the second immunization. Comparing to pTV2 gDsE2, pTV2 ST showed 150%, pTV2 SN2 showed 26%, and pTV2 SN5 showed
10 50%, and further, pTV2 Δ ST showed 144%, pTV2 Δ SN2 did 35%, pTV2 gDs Δ ST showed 104% and pTV2 gDs Δ SN2 did 26% of IFN- γ ELISPOT responses, suggesting that the DNA vaccine including all from core to E2 has similar or superior immunity to a vaccine including
15 only E2 (FIG. 10). These results suggest that the insert length of a foreign gene expressed in a vector could affect the induction of cellular immune response by E2-specific IFN- γ ELISPOT and CTL analyses.

20

We speculated that, first, the longer the insert gene was, the less the expression was (FIG. 5). And the expression level seemed to affect the induction of cellular immune response. But, in fact,
25 cellular immune response was not much differ when

three different vectors expressing the same antigen but having difference in expression level up to 500 times were used, proved by the succeeding experiments by the present inventors. Therefore, the difference in expression level of antigen seemed not to be the major reason. Second, when an antigen having better immunogenicity than E2 was expressed simultaneously with E2, the immune response to E2 became comparatively decreased because of the antigenic interference. According to the earlier reports, non-structural proteins are superior to structural proteins in the induction of cellular immune response. However, after all the experiments followed by the present inventors, cellular immune response to E2 did not decrease even when the additional plasmid expressing the antigen same as pTV2-SN5 was given for the immunization. That is, the simultaneous expression of non-structural proteins and E2 does not decrease cellular immune response against E2. It is a possible guess that the difference in length of polyprotein might affect any time or any part of antigen-presentation during the procedure of the antigen expression within cells. So, further studies on the mechanisms concerning that cellular immune response is affected by the insert length of a

foreign gene expressed in a DNA vector is required.

Example 8: Enhancement of immunogenicity by antigen
engineering of a DNA vaccine-2

5 (FIG. 11: The effect of the elimination of N-terminal
40 amino acids of core on the induction of cellular
immune response to E2)

In this example, the effect of a DNA vaccine on
hepatitis C virus was investigated. The present
10 inventors tried to reconfirm the immunosuppressive
effect of core and at the same time, to provide a way
to avoid the immunosuppressive effect of core. The
present inventors have reported the immunosuppressive
effect of core using antigen presenting cells stably
15 expressing core protein (Lee, *Virology*, 2001,
279:271), and have found through the succeeding
studies on deletion mutant in which immunosuppressive
effect of core can be prevented by eliminating amino
terminal 40 amino acids of core. The embodied
20 example was based on the above preliminary results.

Cellular immune response to E2 was investigated
by using the plasmids 'pTV2-ST' and 'pTV2-SN2', both
expressing core and reporter antigen E2
simultaneously in cytoplasm. In addition, cellular

immune response was also investigated by using the plasmids 'pTV2-Δ ST' and 'pTV2-Δ SN2' in which the same structural proteins are expressed except the elimination of N-terminal 40 amino acids of core.

5 The first immunization was performed with 100 μg of plasmid. 5 weeks later, CD8+ T cellular immune response to E2 was investigated by IFN-γ ELISPOT analysis and cytotoxic T lymphocyte response, using CT26-hgE2t cell line as stimulating cells.

10

As shown in FIG. 11, there was a difference in cellular immune response to E2 between when intact core was expressed and when a core without N-terminal 40 amino acids was expressed. pTV2-Δ ST and pTV2-Δ
15 SN2 showed each 163% (p<0.01) and 134% (p<0.1) increased response, compared with pTV2-ST and pTV2-SN2, as determined by the direct IFN-γ ELISPOT assay. The difference became larger after *in vitro* stimulation for 5 days to 347% (p<0.003) and 800%
20 (p<0.001) respectively, as determined by expanded ELISPOT. Similar results to that of ELISPOT response were observed in cytotoxic T lymphocyte response, in which pTV2-ST and pTV2-SN2 showed 50% and 18% of CTL activity each at the 30 E/T ratio, while pTV2-Δ ST
25 and pTV2-Δ SN2 showed 79% and 54% CTL activity

respectively. The present inventors repeated three more independent tests based on the consideration that the difference in the induced cellular immune response among the above plasmids was statistically
5 insignificant, which reproduced the same results.

The reports on the effect of core on the immunosuppression have been controversial to each other (Liu, 2002 *J Virol* 76:990; Soguero, 2002 *J*
10 *Virol* 76: 9345), which suggests that the effect of core is too small to finish the controversy or is detected only under the specific test conditions. In the present invention, though, the immunosuppressive effect of core was observed by direct ELISPOT, even
15 though the difference was not dramatic, and further confirmed to be increased by expanded IFN- γ ELISPOT analysis. *In vitro* stimulation for 5 days would represent original memory T cells due to the disappearance of effector-stage T cells (Liu, 1997 *J*
20 *Exp Med* 185:251; Susan, 2002 *Nat Rev* 2:251). Thus, the immunosuppressive effect of core is more likely to be distinguished in the stage of memory T cells rather than in effector T cells. However, further studies to analyze the results of the present
25 invention should be followed since it was not fully

probed yet whether the definition on the activation of effector T cells or memory T cells could be applied to the DNA vaccine models as it was. In the succeeding studies by the present inventors, the inventors investigated the effect of core using the same plasmids after the second and the third immunization. As a result, the difference in cellular immune response was decreased or vanished. That is, the effect of core is insignificant or just shown temporarily. According to the reports on the characteristics of HCV antigen specific T cells in the early stage of HCV infection, there is a stage of stunned phenotype, precisely during which division is going on but IFN- γ is not secreted, and after passing through the stage, IFN- γ becomes secreted again (Lechner, 2000 *J Exp Med* 191:1499). Taking this and the results of the embodiments of the present invention together into consideration, core seems to have a temporary immunosuppressive effect.

20

In the example of the present invention, intact core expressed in cytoplasm has immunosuppressive effect, and the effect disappeared when amino terminal 40 amino acids were eliminated. Thus, the present invention provides a method for enhancing the

25

cellular immune response to HCV, even with core keeping amino acids most, by eliminating the immunosuppressive effect of core protein.

5 Example 9: Enhancement of immunogenicity by antigen engineering of a DNA vaccine-3

(FIG. 12: The difference in cellular immune response caused by the existence of transmembrane domain of E2)

10 In the aspect of immunogenicity of E2, the changes of cellular immune response to E2 by transmembrane domain were investigated in this example.

 In order to achieve the object, the plasmid
15 pTV2 gDsE2 expressing E2 only and the other plasmid pTV2 gDsΔ ST expressing Δ Core-E1-E2 all were used. As control groups, pTV2 gDsE2t and pTV2 gDsΔ STt without transmembrane domain of E2 were used. Each plasmid was used for immunization by 100 μg. 5 weeks
20 after the first immunization or 3.4 weeks after the second immunization, cellular immune response to E2 was observed by IFN-γ ELISPOT assay. After the first immunization, CT26-hghE2t cell line was used for the stimulation, and after the second

immunization, peptide pool for E2 was used for the stimulation. The former could stimulate only CD8+ T cells, and the latter could stimulate not only CD8+ T cells but also CD4+ T cells.

5 As shown in FIG. 12, pTV2 gDsE2t and pTV2 gDsΔ STt showed 66% ($p<0.001$) and 50% ($p<0.001$) response respectively on the 5th week from the first immunization, and 40% ($p<0.005$) and 39% ($p<0.004$) each on the 3.4th week from the second immunization,
10 compared with pTV2 gDsE2 and pTV2 gDsΔ ST. Thus, even though whole structural protein was expressed along with E2, cellular immune response to E2 was decreased by the elimination of transmembrane domain of E2.

15

 If T cell epitope was present in transmembrane domain of E2, such result would have been produced. In the embodiment of the present invention, in order to get rid of such possibility, a cell line
20 expressing E2 without transmembrane domain (CT26-hghE2t cell line) was used, and besides, a peptide pool was also prepared by eliminating transmembrane domain of E2. Therefore, the result of the invention has nothing to do with epitope present in
25 transmembrane domain. And thus, the results might be

in the bounds of possibility as follows; First, The extracellular secretion of E2 was induced by the elimination of transmembrane domain. That is, E2 protein stays short in cells as its transmembrane domain is removed. In regard to antigen presenting process, protein is processed by proteasome to be loaded on MHC class I molecule during the protein synthesis, which was then exposed on cell surface through ER by secretory system. But, exceptionally, dendritic cells among antigen presenting cells could stimulate CD8+ T cells by a certain mechanism in which a part of the protein in endosome can be loaded on MHC class I molecule in ER through cross presentation (Heath, 2001 *Nat Rev Immunol* 1:126). It suggests that even a protein after synthesis can join class I antigen presentation pathway. So, the induction of extracellular secretion has an advantage to induce antibody response effectively but a disadvantage not to induce cellular immune response effectively by the short stay in a cell. Second, the difference of a protein structure resulted from the presence or the absence of transmembrane domain might cause changes in many stages related to antigen presenting process. A membrane protein requires a lipid membrane component to keep the structure stable.

The changes in physiochemical characteristics of a protein generated from being produced in cytoplasm to be participated in the antigen presenting process results in the difference observed through antigen
5 presenting process.

The result of this example of the present invention suggested that the elimination of transmembrane domain of E2 protein played a negative role in inducing cellular immune response, so that a
10 DNA vaccine containing transmembrane domain was required for optimal induction of cellular immune response.

There was not much difference in enhancement of
15 immunity by antigen engineering of a DNA vaccine among all the above individual examples, but it was uncertain whether all the substances would have cumulative effect or synergistic effect when they worked altogether for the treatment of a disease.
20 Especially for a virus rapidly producing diverse quasispecies, like HIV or HCV, the valance between immune response and the viral replication is very important to decide whether the viral infection can be cleared or progress to chronic infection. Thus,
25 the above example provides a new approach to enhance

cellular immune response by antigen engineering in HCV DNA vaccine model.

Example 10: Quantification of HCV E2- or core-specific CD4+ T cells secreting IFN- γ and IL-4 (FIG. 13)

The present inventors searched a kind of vaccine and a method including the administration of the same to induce maximum Th1 immune response, a protective immunity against HCV infection. At first, 4 mice from each group were taken to isolate their spleens. 5×10^7 spleen cells were re-suspended in 450 μl of MACS buffer solution (0.5% BSA, 2 mM EDTA in PBS), and then mixed with 50 μl of MACS antibody bead, followed by a reaction at 4°C for 15 minutes. After finishing the reaction, the mixture was washed with 5 ml of MACS buffer solution twice, and then, the samples were re-suspended in 500 μl of MACS buffer solution. Equilibration of MACS mini-column was performed with 500 μl of MACS buffer solution, which was exposed on magnetic field. The sample was loaded on the above column. After the buffer solution was spilled out completely, the column was washed with

MACS buffer solution three times. Magnetic field was removed from the column to elute with 500 μ l of MACS buffer solution. FACS analysis was performed for the obtained cells, from which 90% of those were confirmed to be CD4+ T cells. ELISPOT analysis was performed to quantify those cells secreting IFN- γ and IL-4.

As a result, relatively great numbers of HCV E2- and core-specific CD4+ T cells secreting IFN- γ were produced by DNA priming-rAd boosting method of the invention, compared with other vaccine administrating methods (FIG. 13A and FIG. 13B, G2, $p < 0.001$). When just DNA was injected twice, E2-specific IFN- γ producing CD4+ T cells were hardly observed (FIG. 13A, G1), but those producing core specific IFN- γ were induced as much as in the group treated with rAd twice (FIG. 13B, G1). That was because the core protein itself, unlike E2, could efficiently induce the immunity of CD4+ T cells simply by DNA immunization. On the contrary, from the results of IL-4 ELISPOT analysis, core- and E2-specific CD4+ T cells, synthesizing IL-4 were hardly detected in every group (FIG. 13C and FIG. 13D). That was because a DNA vaccine and an adenovirus

vaccine induced the immune response to Th1 type than to Th2. Considering all the above results, the present inventors confirmed that the DNA priming-rAd boosting method was most effective to induce the CD4+ Th1 immune response than rAd priming-DNA boosting or twice injection of an adenovirus.

Example 11: Investigation of HCV E2-specific cytotoxic T lymphocyte response (FIG. 14)

Each group was treated with a DNA and a recombinant adenovirus vaccine. Two mice from each group were taken to isolate their spleen cells for the investigation of the cytotoxic T lymphocyte immune response. CT26-hghE2t cell line (H-2d restricted) expressing E2 was used to stimulate HCV E2 specific cytotoxic T lymphocytes.

As a result, twice injection of the adenovirus and the DNA priming-rAd boosting method were both proved to be effective without a significant difference between them (FIG. 14, G2 and G3). Although rAd priming-DNA boosting method and twice injection of DNA induced the specific cytotoxic T lymphocyte response, the effect was not like the

above two methods (FIG. 14, G1 and G4). Thus, DNA priming-rAd boosting immunization was most effective to induce the cytotoxic T lymphocyte immune response.

5 Example 12: Immunization of chimpanzees using an
immunogenic plasmid and a recombinant adenovirus and
the challenge with infectious hepatitis C virus (FIG.
15)

10 All the records on the chimpanzees used for the
experiments were presented in Table 1. For the test
group 1 and group 2, 6 mg of HC102 DNA vaccine and 8
mg of HC103 DNA vaccine were dissolved in PBS by the
concentration of 2 mg/ml respectively, and 0.75 ml of
HC102 and 1 ml of HC103 were injected intramuscularly
15 into 4 spots of right and left deltoid muscle and
right and left gluteus maximus, for the group 1 and
group 2 respectively, three times at intervals of 8
weeks. 30 weeks after then, 1×10^{10} pfu of recombinant
adenovirus (rAd gDsΔ ST, rAd gDsNS34, rAd NS5) were
20 dissolved in 1.6 ml of suspension buffer (10 mM Tris,
4% sucrose, 2 mM $MgCl_2$, pH 8.0), which was also
injected intramuscularly into the same 4 spots where
a DNA vaccine was injected by 0.4 ml. 12 weeks after

the final immunization with the recombinant adenovirus, an intravenous injection with HCV-bk challenging inoculum having the concentration of 100 CID₅₀/ml was performed on right femoral vein by 1 ml/chimpanzee.

<Table 1>

Vaccine	Chimpanzee	Name	Gender	Date of Birth	Mother	HCV infection of mother	Infection period of mother	Exposure to HBV	Exposure to HCV
Control	404	Chop Suey	F	94.09.12	Spring Roll	Recovered	96.9-96.12	-	-
	406	Noli	F	94.11.25	Annie	Recovered	89.8-90.2	-	-
HC102/rAd HC102	376	Sabel	F	91.08.25	Mabel	-		-	-
	393	Sally	F	92.11.02	Lucy	-		-	-
	400	Pasiway	M	94.05.09	Pasimani	-		-	-
HC103/rAd HC103	381	Troy	F	91.10.19	Helen	-		-	-
	397	Root Bear	F	93.12.05	Sarsaparilla	Recovered	88.8-88.11	-	-
	402	Lawrence	M	94.07.02	Juno	-		-	-

Example 13: Isolation of the immunized cells from chimpanzees

Blood was taken from right femoral vein of chimpanzee with 10 ml heparinized vacutainer. PBMC
5 (peripheral blood mononuclear cell) was isolated for the investigation of cellular immune response.

Blood in the two heparinized vacutainers was diluted using RPMI-1640 medium to make a total volume 30 ml, which was then transferred by pipette into 50
10 ml conical tube containing 15 ml of lymphocyte separation medium (Cellgro Mediatech, Cat# 25-072-CV) to let them form each layer. Centrifugation was performed with the mixture at room temperature with 1800 RPM for 20 minutes with a centrifuge (Eppendorf
15 refrigerated tabletop centrifuge). Plasma in the upper layer was removed. The below PBMC layer was separated carefully with a pipette, and then transferred into a 50 ml fresh conical tube. The layer was diluted with RPMI-1640 medium to make a
20 total volume 50 ml. Centrifugation was performed again under the same speed and temperature for 10 minutes to eliminate supernatant. The process was repeated two more times, and the separated PBMC was washed completely. The product was diluted with 0.4%

tryphan blue (Sigma, Cat# 8154) to quantify the cells with a hemacytometer, which was later used for the investigation of cellular immune response.

5 Example 14: IFN- γ ELISPOT analysis using chimpanzee
 PBMC

IFN- γ ELISPOT analysis was performed using PBMC obtained from the immunized chimpanzees by following the manufacturer's instruction. IFN- γ ELISPOT kit
10 (MABTECH Co., Cat# M34201-H) was used for this invention. Precisely, antibody (1-D1K) to IFN- γ was diluted with PBS by the concentration of 5 μ g/ml, which was plated on a 96 well plate (Millipore, 0.45m, Cat# MAHAS4510, Bedford, MA) by 50 μ l/well. The
15 plate was left at room temperature for over 12 hours. The remaining antibody solution was removed by suction. Then, the plate was washed with PBS twice. Animal cell culture medium (RPMI 1640 containing 50 units/ml penicillin, 50 μ g/ml streptomycin, 50 μ M β
20 -mercaptoethanol, 100 M MEM non-essential amino acid, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 20 Units/ml recombinant hIL-2 and 1% ABS (human AB serum, Valley Biomedical, Lot# A20124)) was added to each well by 200 μ l. The plate was left again at 37°C

for over 2 hours, followed by suction. The separated PBMC was plated thereto by 3×10^5 cells/well. In order to investigate HCV-specific cellular immune response, peptide pool was added by $1 \mu\text{g/ml}$ per each peptide. For the positive control, phytohemagglutinin (PHA) was treated by $2.5 \mu\text{g/ml}$. The 96 well plate was put in a 37°C 5% CO_2 incubator and left for 18 hours without agitation. The contents of the 96 well plate were thrown off. And
10 the plate was washed with washing solution (PBS-T, 0.1%) containing 0.1% tween 20 (Sigma, Cat# D8654) four times. Biotin-conjugated mAb (7-B6-1 biotin) was diluted with blocking buffer prepared by adding 1% BSA to washing solution to make the final
15 concentration of $1 \mu\text{g/ml}$, which was added to each well by $50 \mu\text{l}$, leading to a reaction for 2 hours. The plate was washed with the washing solution (PBS-T, 0.1%) four times and filled with streptavidin-HRP solution that was diluted by the blocking buffer at
20 the ratio of 100:1. Reaction was followed for an hour. After finishing the reaction, AEC substrate solution was used to induce color development. The color development was stopped by using tap water when the spot with expected size was observed (5-10
25 minutes). The 96-well plate was dried at room

temperature. And the cells secreting IFN- γ were quantified by ELISPOT reader.

The above experiment was carried out on the 2nd week after boosting with a recombinant adenovirus. As a result, even though there was a slight difference between individuals, over 1000 IFN- γ secreting cells per a million PBMCs were detected in 4 of 6 chimpanzees. Even much less, the rest 2 chimpanzees produced 400-600 HCV antigen specific immune cells. No difference was observed between experimental group 1 and group 2. Also, over 1200 IFN- γ secreting cells per a million immune cells were found in #404 chimpanzee, a control group of chimpanzee. That was not because the examples of the present invention reflected non-specific immune response, but because the target was exposed on HCV under the infectious dose once in the past. In that case, viremia was not developed, but the induced cellular immune response was memorized long enough to show a specific response later (Shata MT, 2002, 9th *International Meeting on HCV and Related Viruses*, P-215), which is consistent with the recent test results of New York Blood Center. Thus, there is no doubt about that the methods of the present invention

effectively induce cellular immune response in large animal model.

Example 15: Quantification of IFN- γ secretion by CD4+

5 T cells and T cell proliferation assay in chimpanzees

2 weeks after boosting with a recombinant adenovirus, PBMC was isolated by the procedure mentioned above to quantify HCV antigen specific IFN- γ secretion by CD4+ T cells. IFN- γ ELISA kit (BD pharmingen Co.) was used in this example. PBMC, separated from chimpanzee, was diluted with growth medium (RPMI-1640 containing 50 units/ml penicillin, 50 μ g/ml streptomycin, 50 μ M β -mercaptoethanol, 100 M MEM non-essential amino acid, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 10% ABS (human AB serum, Valley Biomedical, Lot# A20124)) in the absence of human recombinant IL-2, and then distributed in a round 96 well plate (2×10^5 cells/well). And stimulation with specific antigens was given. PHA having the final concentration of 2.5 μ g/ml was selected as a positive control. As an HCV specific antigen, 5 μ g/ml of recombinant GST-Core, GST-NS3, and NS5 proteins were used, and a recombinant human SOD (superoxide dismutase) was used

for a negative control. The plate was cultured for 5 days in a 37°C, 5% CO₂ incubator. On the 5th day, 100 µl of culture supernatant was taken for the quantification of IFN-γ using IFN-γ ELISA kit (BD pharmingen Co.) by following the standard protocol of the manufacturer. On the other hand, ³H-thymidine was added to the remaining cells on 96-well plate by 1 µCi/well to investigate CD4⁺ T cell proliferation. 18 hours after adding ³H-thymidine, cells were harvested on a glass filter paper (Wallac, Finland, Cat# 1205-401) using an automatic cell harvester (Micro 96 Cell harvester, Model 11055, Skatron Instruments Inc, VA), after which the amount of ³H-thymidine incorporated was measured by a liquid scintillation counter (Microbeta Plus 1450 Liquid Scintillation Counter, Wallac, Finland). The stimulation index was calculated by dividing the amount of HCV antigen-specific radioactivity (cpm) incorporation by SOD-specific radioactivity (cpm) incorporation.

20

As a result of this example in which IFN-γ secretion by CD4⁺ T cell was investigated, a specific response to HCV nonstructural protein was observed in chimpanzees administered with the vaccine, which was, though, very weak in the control group. This result

25

coincides with the earlier report that the nonstructural protein among many antigens of HCV could induce cellular immune response better (Missale, 1996, *J Clin Invest* 98: 706). IFN- γ secretion in experimental group 1 was greater than that in group 2.

It was also confirmed from the observation on CD4+ T cell proliferation that HCV nonstructural protein specific stimulation index was 20 at average in the vaccine-administered group. T-cell proliferative response showed similar result to IFN- γ secretion by CD4+ T cells in general, but just one chimpanzee (#406) in the control group showed the similar level of an antigen specific response to chimpanzees in the vaccinated groups. That was also resulted from the previous exposure as explained above.

Therefore, it was confirmed by the examples of the invention that the vaccine and the method of the present invention could induce cellular immune response effectively even in large animal model like chimpanzee.

Example 16: Quantification of hepatitis C virus in chimpanzees

After the challenge with infectious hepatitis C virus, how cellular immune response induced by the present invention controls hepatitis C virus replication was investigated by measuring the amount of the virus in blood. The plasma taken from the blood of chimpanzees was used for the quantification using real time RT-PCR at New York Blood Center. And quantitative PCR was performed. Taking the standard HCV RNA, which was quantified already, as a standard, 10 μl of plasma sample was mixed with RT mix (4 μl of 5X RT buffer from Gibco, 1 μl of 100 mM DTT, 1 μl of 10 mM dNTP, 0.5 μl of 100 μM HCV-R primer (SEQ. ID. No 37), 0.1 μl of 4 U/ μl RNasin, 0.1 μl of 200 U/ μl M-MLV and 3.3 μl of H_2O) containing 10 μl of M-MLV reverse transcriptase. Then, reverse transcription was performed at 42°C. 30 μl of PCR mix (3 μl of PCR buffer, 4 μl of 200 mM MgCl_2 , 0.05 μl of 100 μM HCV-F primer (SEQ. ID. No 38), 1 μl of 200 ng/ μl CMB3 fluorescent probe (5-FAM SEQ. ID. No 39 DABCYL-3), 0.25 μl of 5 U/ μl TaqGold polymerase, 21.7 μl of H_2O) was added to the above RT product and PCR was performed using a PCR machine (PE 7700, Perkin Elmer).

The condition of the PCR was as follows: preheating at 95°C for 10 minutes, at 95°C for 30 seconds, at 55°C for 60 seconds and at 72°C for 30 seconds, making 45 cycles in total. On completion, the amount of the virus RNA was calculated with software provided by PE 7700 PCR machine.

Quantification was carried out on 0, 2nd, and 4th week from the challenge with 100 CID₅₀ of infectious hepatitis C virus. Thus, the results reflected the virus replication in the early stage of infection. The amount of the virus was 10^{5.45} and 10⁶ each on the 2nd and 4th week in the control. This result reflected the infectivity of the infectious virus of the invention, and the measured value was good enough for the comparison with the experimental group. On the other hand, 5 of the chimpanzees in the experimental group administered with a vaccine were confirmed not to have virus on the 2nd week. The virus levels of #400 and #381 were near the detection limit (10^{2.95}) of the assay, so that re-test was performed, resulting in no detection of the virus. However, the level on the 4th week was a little higher than the detection limit. The test results were presented as a mean value. There was as 100 times as difference

in the average virus number between the chimpanzees of the experimental group and those of the control. As for the chimpanzees in the control group (naïve) in which immune response to HCV was not induced, the virus began to be detected from 1.02-1.14 week after the challenge, and the maximum virus content ($10^{5.8}$) was observed on the 6th week (Prince AM, 2002, 9th International Meeting on HCV and Related Viruses, P-259). As for the chimpanzees in which the protective immunity to HCV was memorized by experiencing the infection already, the maximum value of the virus was dropped 10-100 times after the re-challenge, compared with the value of the first infection. In addition, the virus stayed short and the time point of the maximum virus became advanced (Bassett SE, 2001, *Hepatology* 33:1479-1487). Taking all the results of the embodiments of the invention into consideration, the vaccine and the method of the present invention are believed to not only induce antigen specific cellular immunity but also control HCV replication during the acute phase of infection. Though, in order to make sure that the protective effect against the infection in the early stage can go far to inhibit the chronic infection, the continuous observation after the infection was required for a

long while. The present invention confirmed that the vaccine of the present invention could induce the protective immunity to the infectious virus in chimpanzees, the only available test animal for HCV.

5

As a result, the vaccine of the present invention developed by the antigen engineering in small animal, and the DNA priming-recombinant adenovirus boosting method of the present invention, which enhances the Th1 immune response, can induce cellular immune response effectively in chimpanzee model resembled in human most, and further the induced cellular immune response can function as a protective immunity against HCV infection.

15

INDUSTRIAL APPLICABILITY

As explained hereinbefore, the plasmid of the present invention used a whole HCV gene as a vaccine to induce multi-epitope-specific cellular immune response. And after determining the optimum size, the whole gene was divided into 3 parts to prepare a DNA vaccine having efficient immunogenicity. In addition, amino terminal 40 amino acids of core were deleted to eliminate its immunosuppressive function,

20

but transmembrane domain of E2 was still included to produce a vaccine having greater immunogenicity. On consideration of Th1 immune response, which is important protective immunity to HCV, the vaccination regimen using DNA priming-recombinant adenovirus boosting was selected, which showed higher CD4+ T cell response than when DNA priming and recombinant adenovirus boosting were used individually. The present inventors have observed that the efficient cellular immune response was induced in chimpanzees, the only available test animal for HCV, and the infectious hepatitis C virus was effectively controlled in the early stage of infection by the vaccine of the present invention. Therefore, the vaccine of the present invention can be effectively used as a vaccine for hepatitis C virus.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as

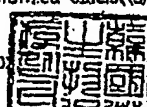
set forth in the appended claims.

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

To: Young Chul Sung
Department of Life Science,
Pohang University of Science and Technology,
Pohang 790-784, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : pGX10 gDs/ST	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10415
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Aug. 29, 2002. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361 221, Yurim B/D Hongje-1-dong, Seodaeamun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) : Data: Sep. 5, 2002 

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BP/4


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**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

To: Young Chul Sun
Department of Life Science,
Pohang University of Science and Technology,
Pohang 790-784, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : pGX10 NS5	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10416
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Aug. 29, 2002. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 351-221, Yurim D/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) : Date: Sep. 5, 2002 

¹ Where Rule 6.4(d) applies, such date is the date on which the status of International depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of International depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the International depositary authority.

Form EP/4


Sole page

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

To: Young Chul Sung
Department of Life Science,
Pohang University of Science and Technology,
Pohang 790-784, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
Issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : PGX10 NS34	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10417
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Aug. 29, 2002. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Ilongje-1-dong, Seodaejeon-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of any () : Date: Sep. 5, 2002 

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BR/1


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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: Young Chul Sung
Department of Life Science,
Pohang University of Science and Technology,
Pohang 790-784, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : Ad-gDs4ST	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10418
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Aug. 29, 2002. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 351-221, Yurim B/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: Sep. 5, 2002 <div style="text-align: center; margin-top: 10px;">  </div>

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BP/4


Sole page

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: Young Chul Sung
Department of Life Science,
Pohang University of Science and Technology,
Pohang 790-784, Korea

RECEIPT IN THE CASE OF AN ORIGINAL,
Issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : rAd-NS5	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10419
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Aug. 29, 2002. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: Sep. 5, 2002 <div style="text-align: right; margin-top: 10px;">  </div>

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BP/4

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To, Young Chul Sung
Department of Life Science,
Pohang University of Science and Technology,
Pohang 790-784, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : rAd-gDsNS34	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10420
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
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IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 351, 221, Yurim E/D Hongje 1-dong, Seodae-mun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: Sep. 5, 2002

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What is claimed is

1. A DNA vaccine including plasmid containing 2-6 kb
of the total antigen gene of hepatitis C virus
5 (HCV).
2. The DNA vaccine as set forth in claim 1, wherein
the DNA vaccine includes plasmid containing 2-4
kb of the total antigen gene of HCV.
- 10 3. The DNA vaccine as set forth in claim 1, wherein
the DNA vaccine includes all of the 1st plasmid
containing core, E1 and E2 genes, the 2nd plasmid
containing NS3 and NS4 genes, and the 3rd plasmid
15 containing NS5 gene.
4. The DNA vaccine as set forth in claim 3, wherein
the 1st plasmid contains core in which 35-40 amino
acids of N-terminal region are eliminated.
- 20 5. The DNA vaccine as set forth in claim 4, wherein
the 1st plasmid contains core in which 40 amino
acids of N-terminal region are eliminated.
- 25 6. The DNA vaccine as set forth in claim 3, wherein

the E2 gene of the 1st plasmid contains transmembrane domain of E2 protein.

7. The DNA vaccine as set forth in claim 3, wherein the 1st plasmid contains a base sequence represented by SEQ. ID. No 50.

8. The DNA vaccine as set forth in claim 7, wherein the 1st plasmid is pGX10 gDsΔ ST (Accession No: KCCM 10415).

9. The DNA vaccine as set forth in claim 3, wherein the 2nd plasmid contains a base sequence represented by SEQ. ID. No 51.

10. The DNA vaccine as set forth in claim 9, wherein the 2nd plasmid is pGX10 NS34 (Accession No: KCCM 10417).

11. The DNA vaccine as set forth in claim 3, wherein the 3rd plasmid contains a base sequence represented by SEQ. ID. No 52.

12. The DNA vaccine as set forth in claim 11, wherein the 3rd plasmid is pGX10 NS5 (Accession No: KCCM

10416).

13. The DNA vaccine as set forth in claim 3, wherein
the 1st plasmid contains a base sequence
5 represented by SEQ. ID. No 50, the 2nd plasmid
contains a base sequence represented by SEQ. ID.
No 51, and the 3rd plasmid contains a base
sequence represented by SEQ. ID. No 52.

10 14. The DNA vaccine as set forth in claim 13, wherein
the 1st plasmid is pGX10 gDsΔ ST (Accession No:
KCCM 10415), the 2nd plasmid is pGX10 NS34
(Accession No: KCCM 10417), and the 3rd plasmid is
pGX10 NS5 (Accession No: KCCM 10416).

15

15. The DNA vaccine as set forth in claim 14, wherein
the pGX10 hIL-12m is additionally contained.

16. A recombinant adenovirus vaccine including an
20 adenovirus containing 2-6 kb of total antigen
gene of HCV.

17. The recombinant adenovirus vaccine as set forth
in claim 16, wherein the recombinant adenovirus
25 vaccine includes an adenovirus containing 2-4 kb

of total antigen gene of HCV.

18. The recombinant adenovirus vaccine as set forth in claim 16, wherein the recombinant adenovirus vaccine includes all of the 1st adenovirus containing core, E1 and E2 genes, the 2nd adenovirus containing NS3 and NS4 genes, and the 3rd adenovirus containing NS5 gene.
10. 19. The recombinant adenovirus vaccine as set forth in claim 18, wherein the 1st adenovirus contains core in which 35-40 amino acids of N-terminal region are eliminated.
- 15 20. The recombinant adenovirus vaccine as set forth in claim 19, wherein the 1st adenovirus contains core in which 40 amino acids of N-terminal region are eliminated.
- 20 21. The recombinant adenovirus vaccine as set forth in claim 18, wherein the E2 gene of the 1st adenovirus contains transmembrane domain of E2 protein.
- 25 22. The recombinant adenovirus vaccine as set forth

in claim 18, wherein the 1st adenovirus contains a
base sequence represented by SEQ. ID. No 50.

23. The recombinant adenovirus vaccine as set forth
5 in claim 22, wherein the 1st adenovirus is rAd gDs
Δ ST (Accession No: KCCM 10418).

24. The recombinant adenovirus vaccine as set forth
in claim 18, wherein the 2nd adenovirus contains a
10 base sequence represented by SEQ. ID. No 54.

25. The recombinant adenovirus vaccine as set forth
in claim 24, wherein the 2nd adenovirus is rAd gDs
NS34 (Accession No: KCCM 10420).

15

26. The recombinant adenovirus vaccine as set forth
in claim 18, wherein the 3rd adenovirus contains a
base sequence represented by SEQ. ID. No 52.

20 27. The recombinant adenovirus vaccine as set forth
in claim 26, wherein the 3rd adenovirus is rAd NS5
(Accession No: KCCM 10419).

28. The recombinant adenovirus vaccine as set forth
25 in claim 18, wherein the 1st adenovirus contains a

base sequence represented by SEQ. ID. No 50, the
2nd adenovirus contains a base sequence
represented by SEQ. ID. No 54, and the 3rd
adenovirus contains a base sequence represented
5 by SEQ. ID. No 52.

29. The recombinant adenovirus vaccine as set forth
in claim 28, wherein the 1st adenovirus is rAd gDs
Δ ST (Accession No: KCCM 10418), the 2nd adenovirus
10 is rAd gDs NS34 (Accession No: KCCM 10420), and
the 3rd adenovirus is rAd NS5 (Accession No: KCCM
10419).

30. A vaccine administrating method characterized by
15 enhancing the protective immunity to HCV by
boosting with the recombinant adenovirus vaccine
of claim 16 after priming with the DNA vaccine of
claim 1.

20 31. The vaccine administrating method as set forth in
claim 30, wherein the priming frequency of the
DNA vaccine is 4-5.

32. The vaccine administrating method as set forth in
25 claim 31, wherein the priming frequency of the

DNA vaccine is 3.

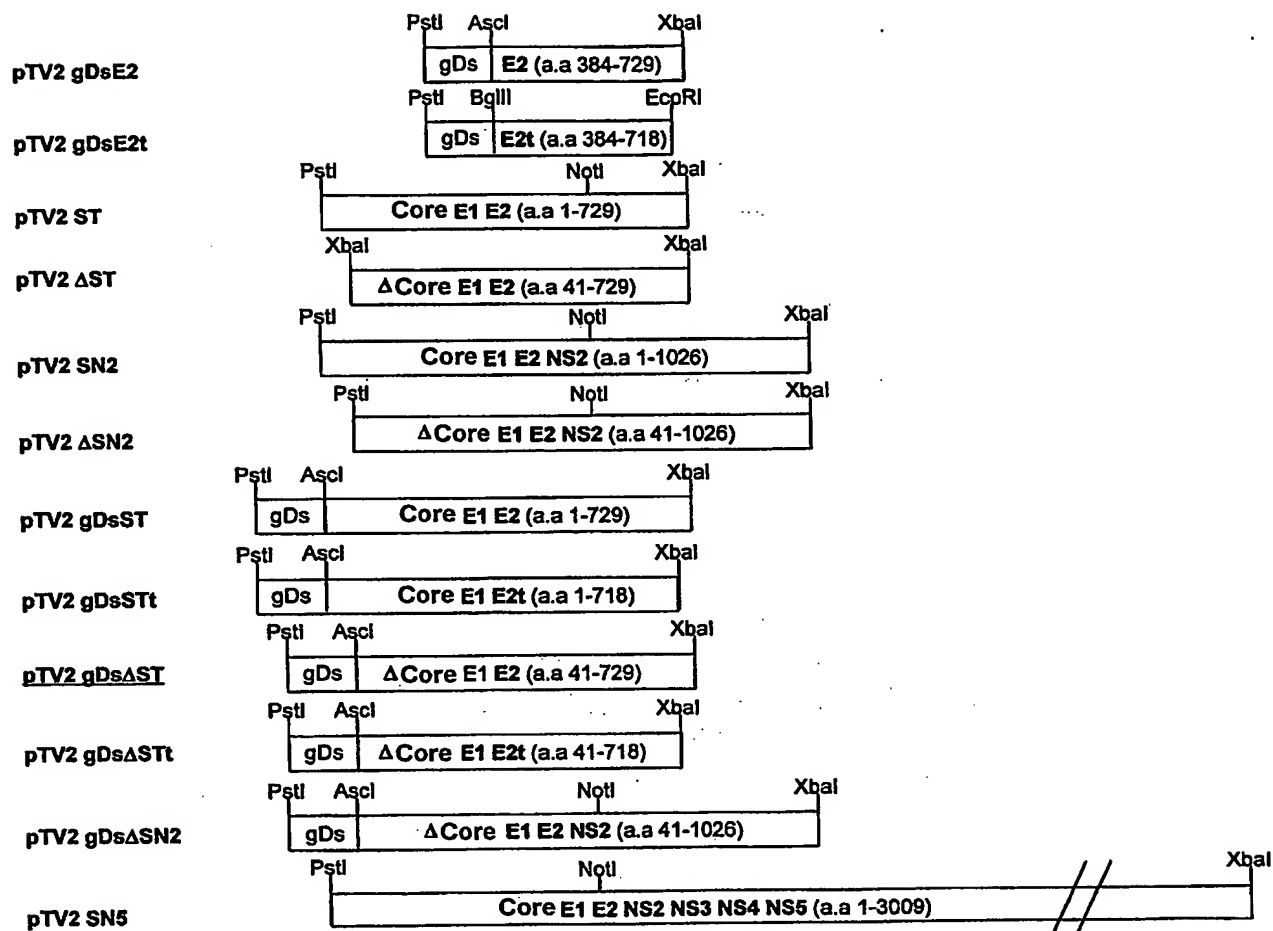
33. A vaccine administrating method characterized by enhancing the protective immunity to HCV by boosting with the recombinant adenovirus vaccine of claim 28 once after priming with the DNA vaccine of claim 13 three times.
34. A method to enhance the protective immunity to HCV by increasing CD4+ Th1 immune response by boosting with the recombinant adenovirus vaccine of claim 16 after priming with the DNA vaccine of claim 1.
35. A method to enhance the protective immunity to HCV by increasing CD4+ Th1 immune response by boosting with the recombinant adenovirus vaccine of claim 28 once after priming with the DNA vaccine of claim 13 three times.
36. A method for the prevention and the treatment of hepatitis C, which is characterized by boosting with the recombinant adenovirus vaccine of claim 16 after priming with the DNA vaccine of claim 1.

37. A method for the prevention and the treatment of hepatitis C, which is characterized by boosting with the recombinant adenovirus vaccine of claim 28 once after priming with the DNA vaccine of claim 13 three times.

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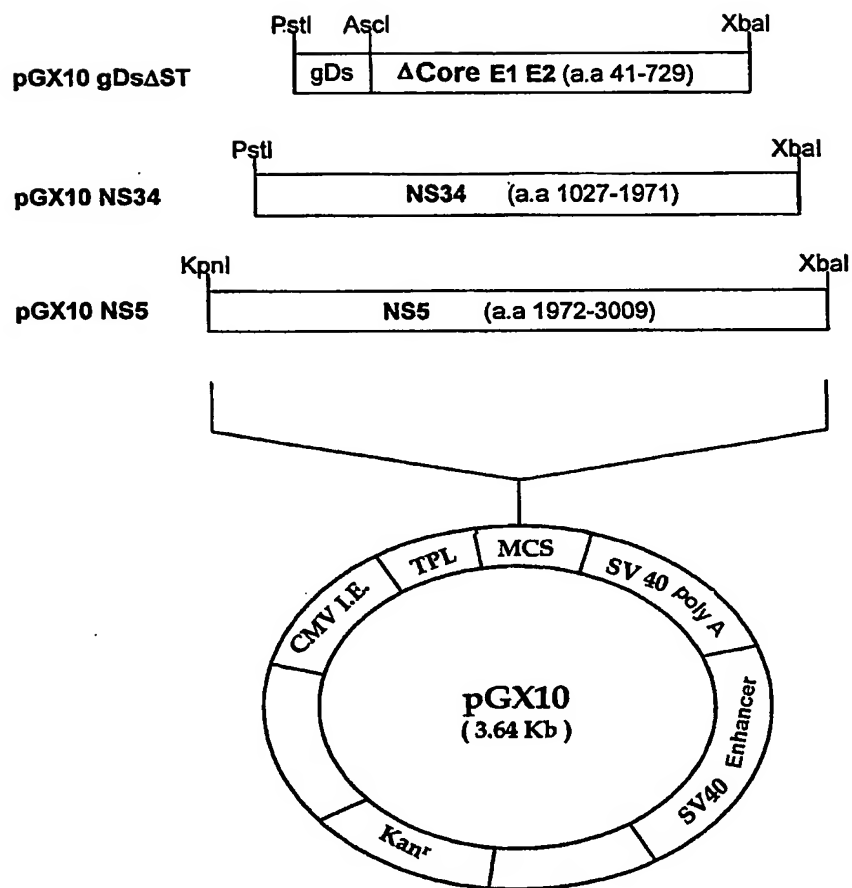
FIGURES

FIG. 1



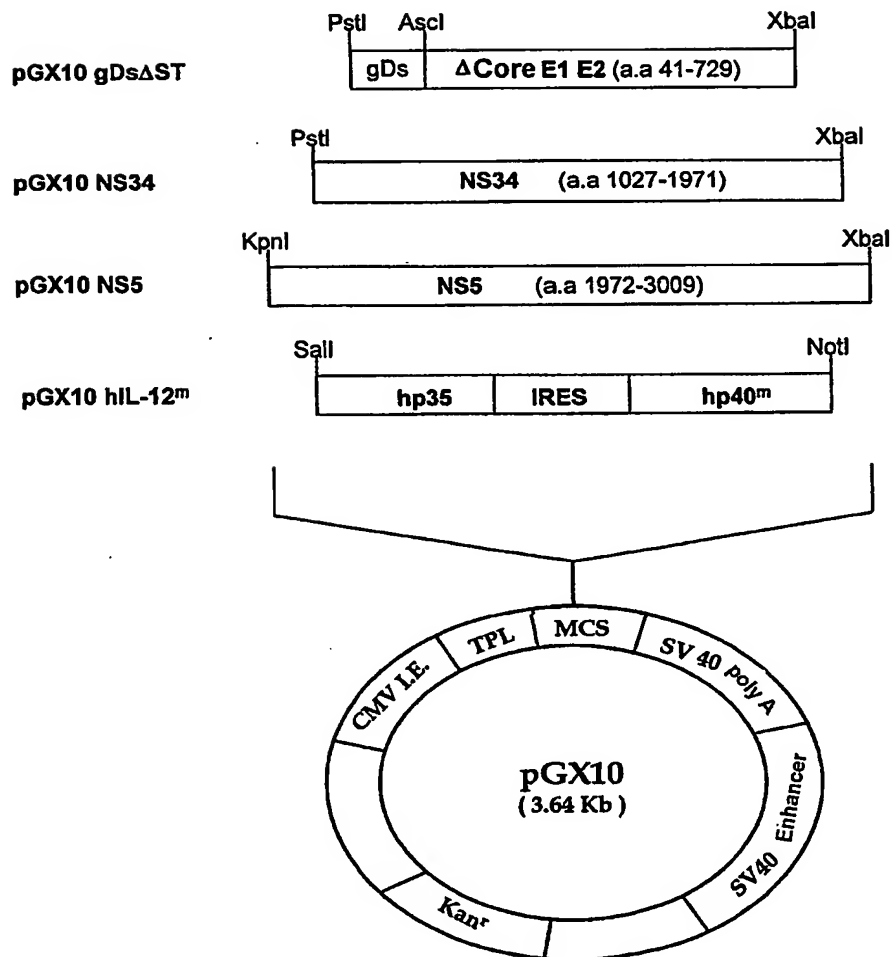
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FIG. 2

HC102

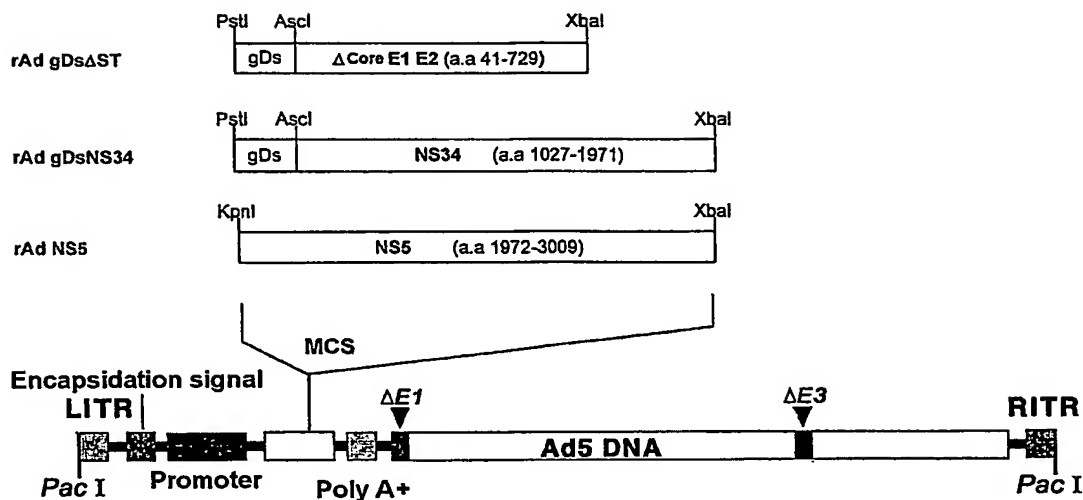
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FIG. 3

HC103

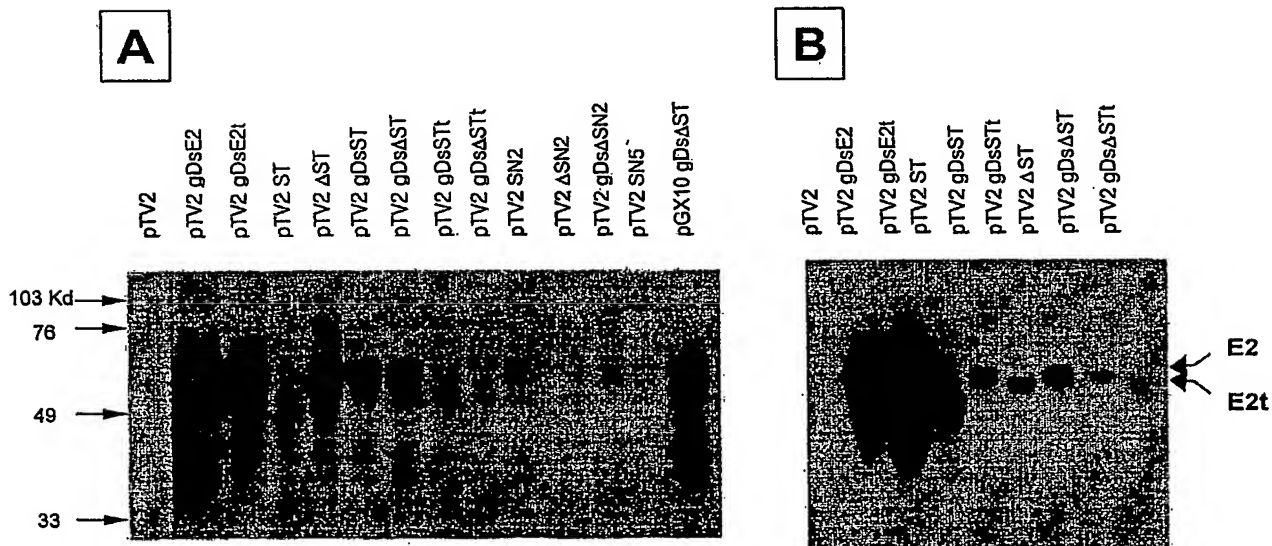
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FIG. 4



rAd HC102

FIG. 5



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FIG. 6

COS-7

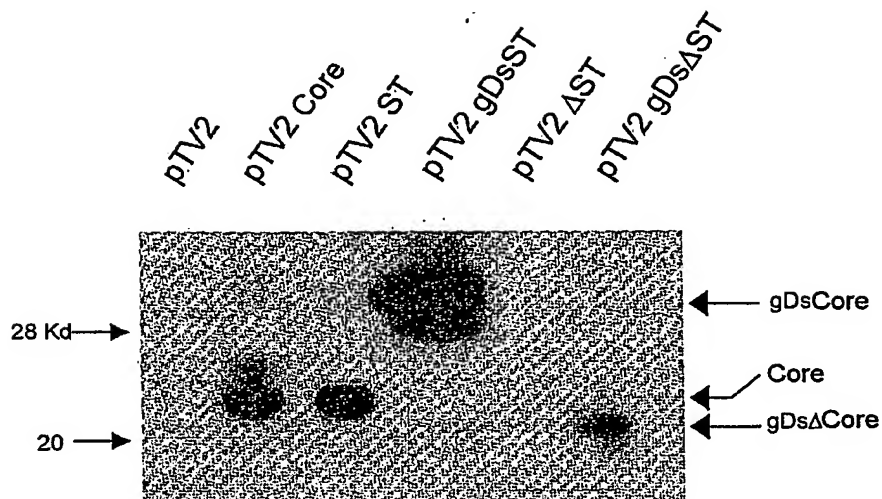
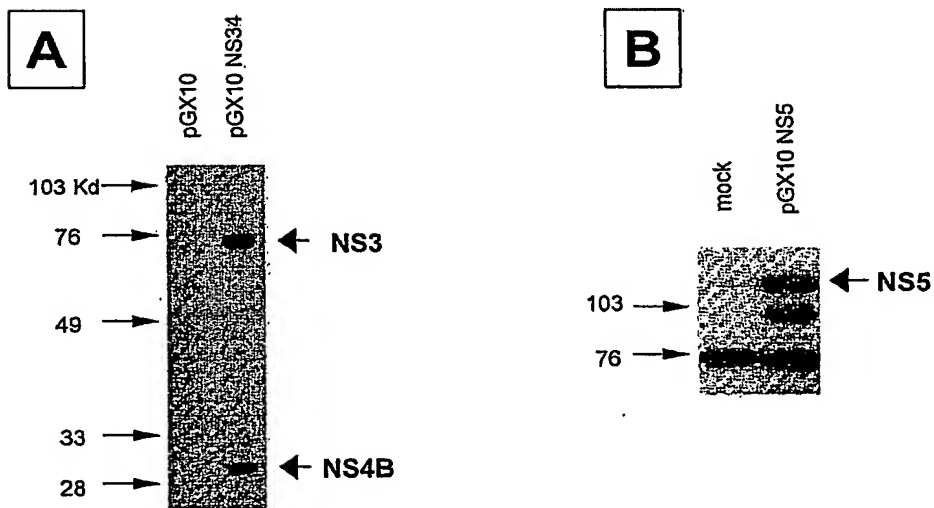


FIG. 7

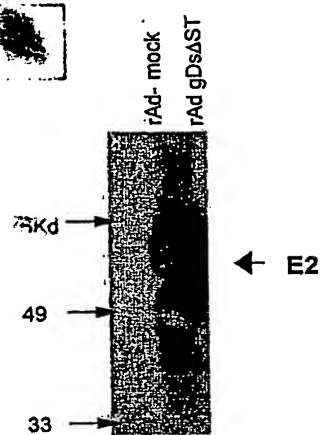
COS-7



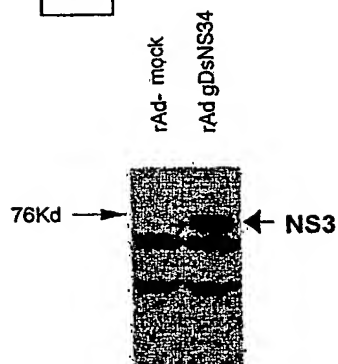
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FIG. 8

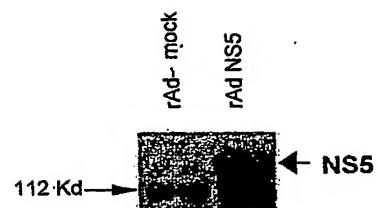
3A



B



C



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FIG. 9

Optimization of insert size

(5 weeks after immunization)

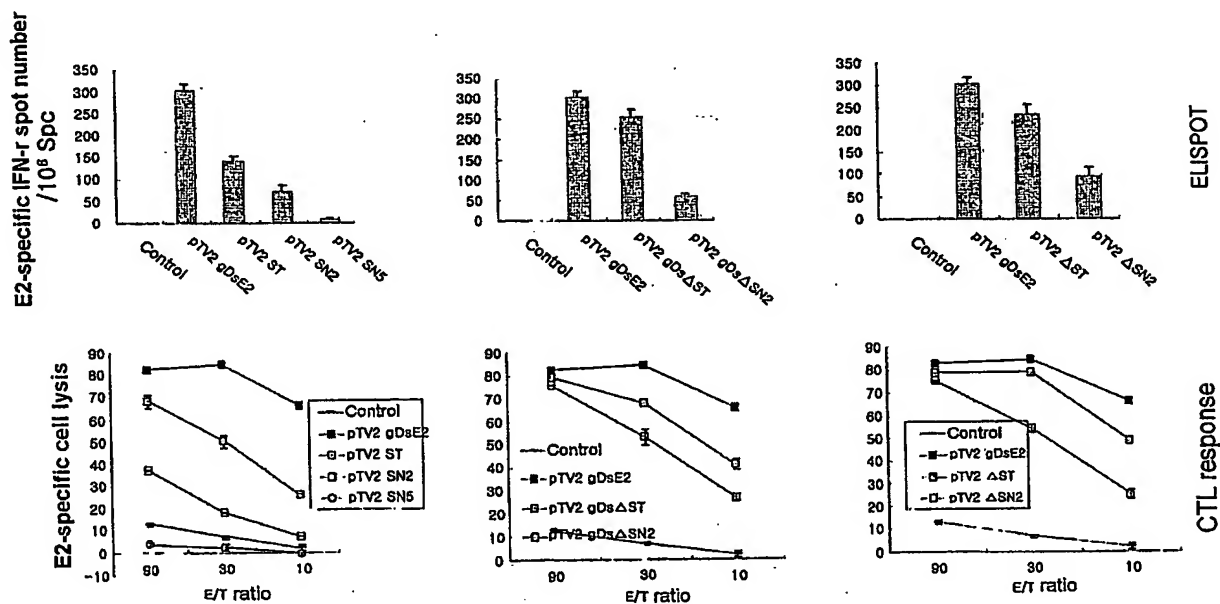
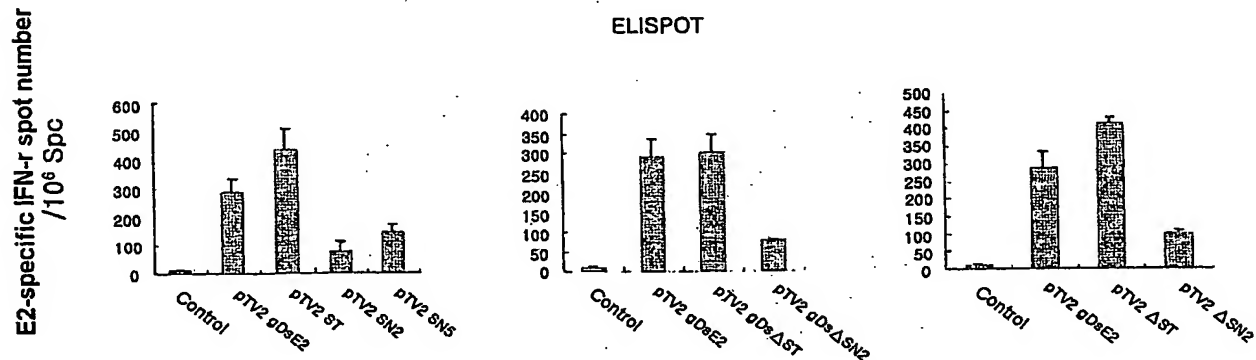
E2 specific IFN-r ELISPOT & CTL response
target cell : 2×10^4 CT26-hghE2t/well

FIG. 10

Optimization of insert size

(3, 4 weeks after boosting)

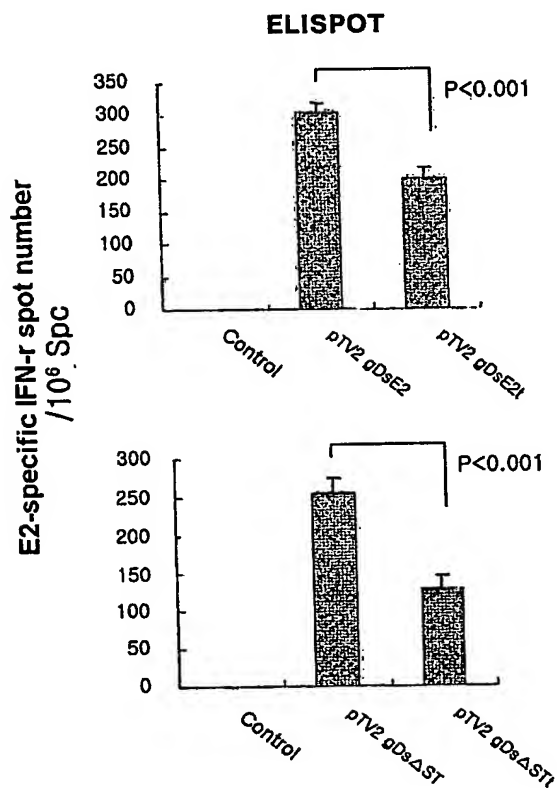
E2 specific IFN-r ELISPOT & CTL response
target cell : 2×10^4 CT26-hghE2t/well

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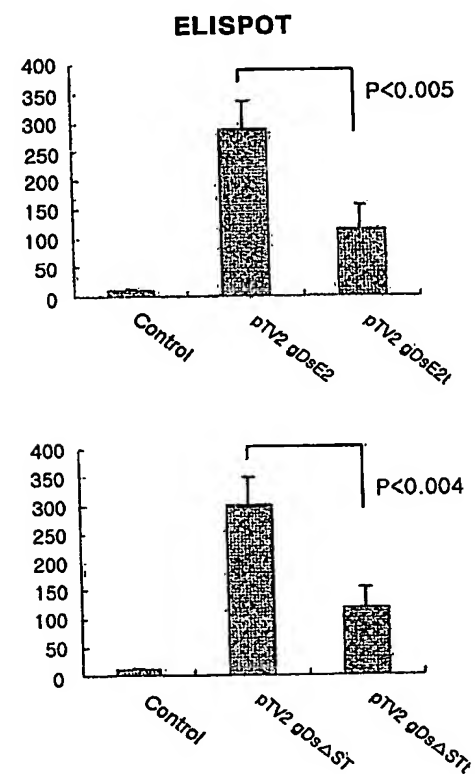
FIG. 12

Truncation of E2 TM domain

5 weeks after immunization

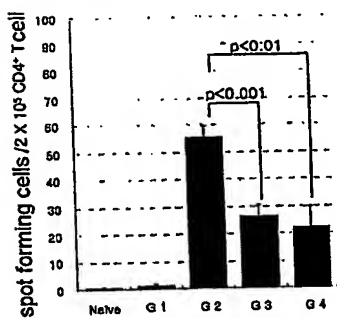
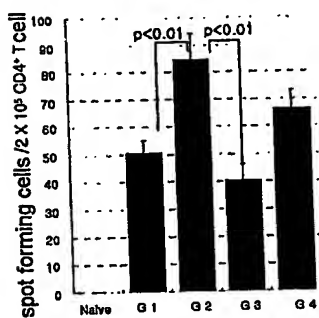


3.4 weeks after boosting

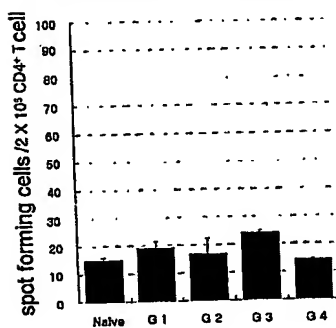


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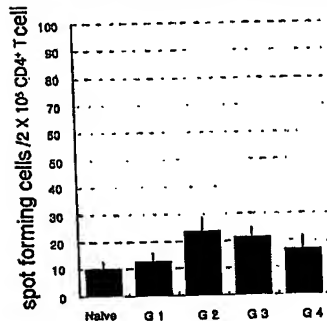
FIG. 13

a. E2 specific IFN- γ ELISPOTb. core specific IFN- γ ELISPOT

c. E2 specific IL-4 ELISPOT



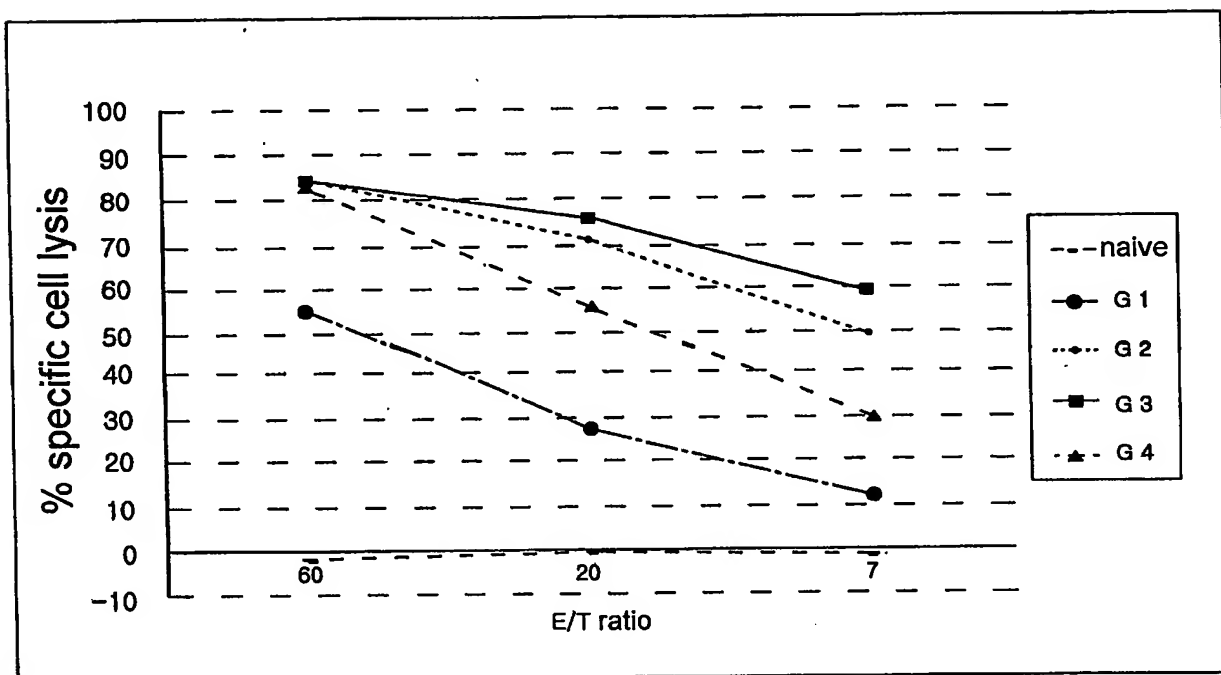
d. core specific IL-4 ELISPOT



naive : saline injected control
 G 1: pGX10 gDsΔST + pGX10
 mL-12mutant injection →
 pGX10 gDsΔST + pGX10
 mL-12mutant injection
 G 2: pGX10 gDsΔST + pGX10
 mL-12mutant injection →
 rAd gDsΔST injection
 G 3: rAd gDsΔST injection →
 rAd gDsΔST injection
 G 4: rAd gDsΔST injection →
 pGX10 gDsΔST + pGX10
 mL-12mutant injection

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FIG. 14

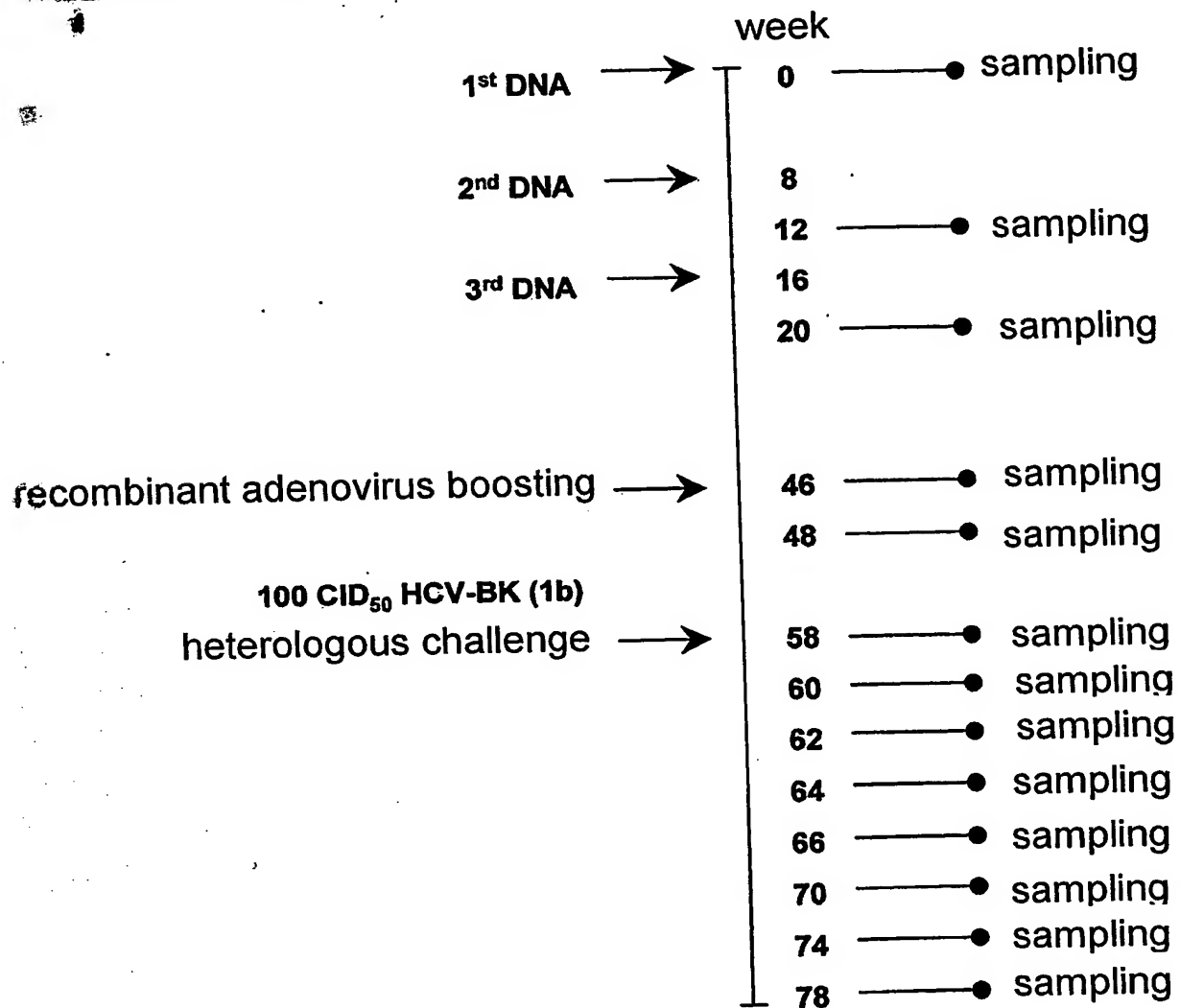


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FIG. 15

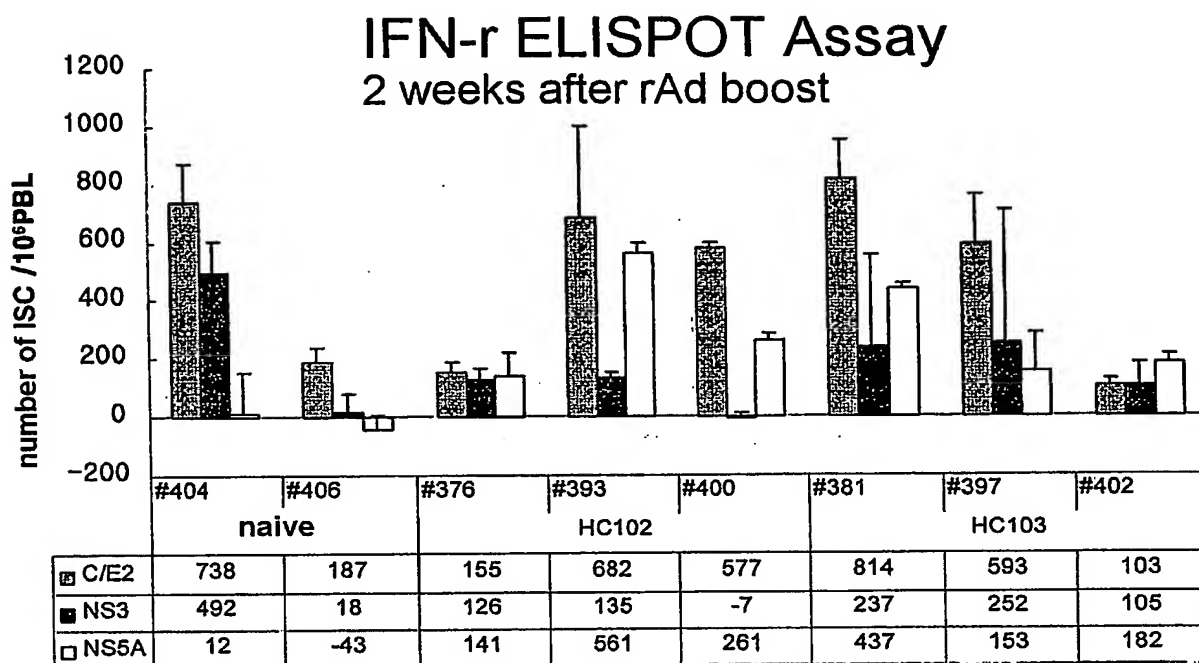
Schedule)

DNA prime/ rAd boost



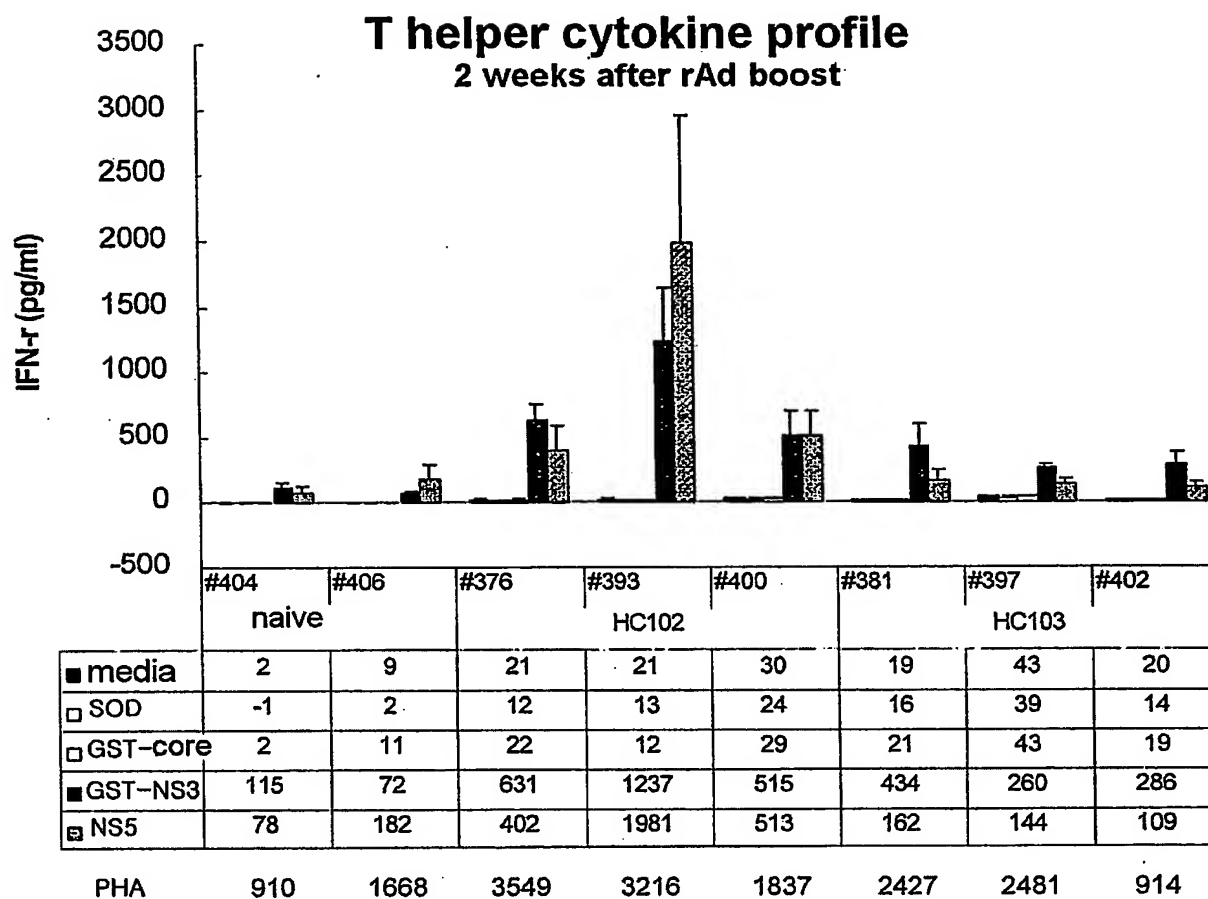
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FIG. 16



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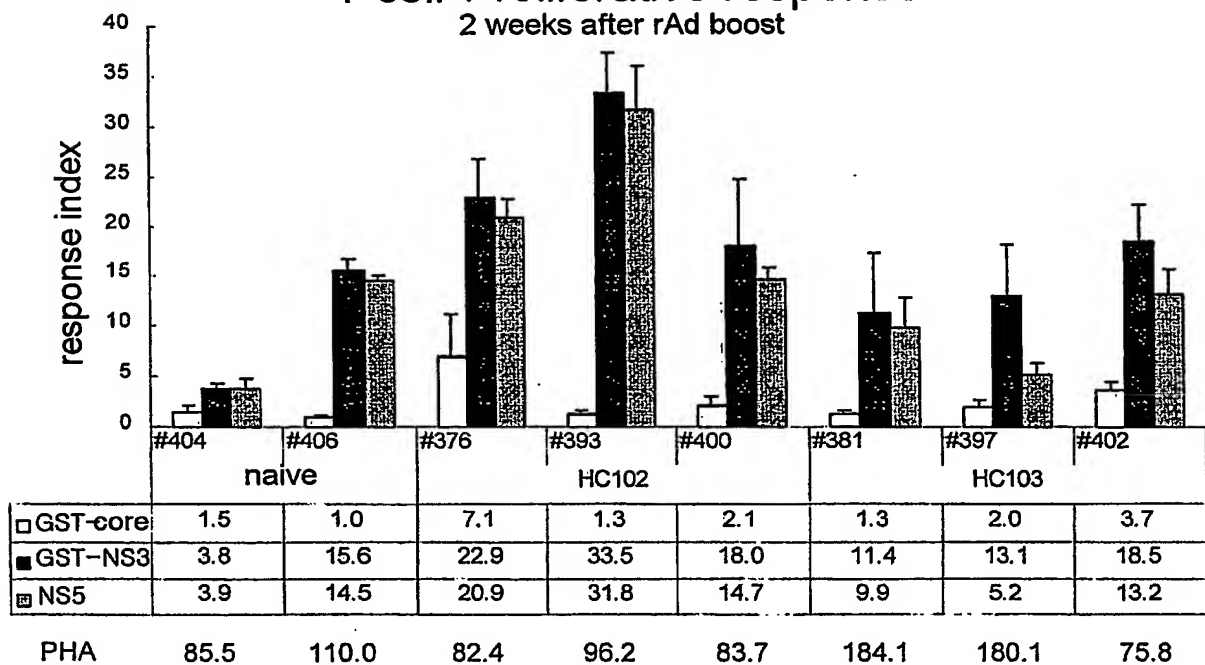
FIG. 17



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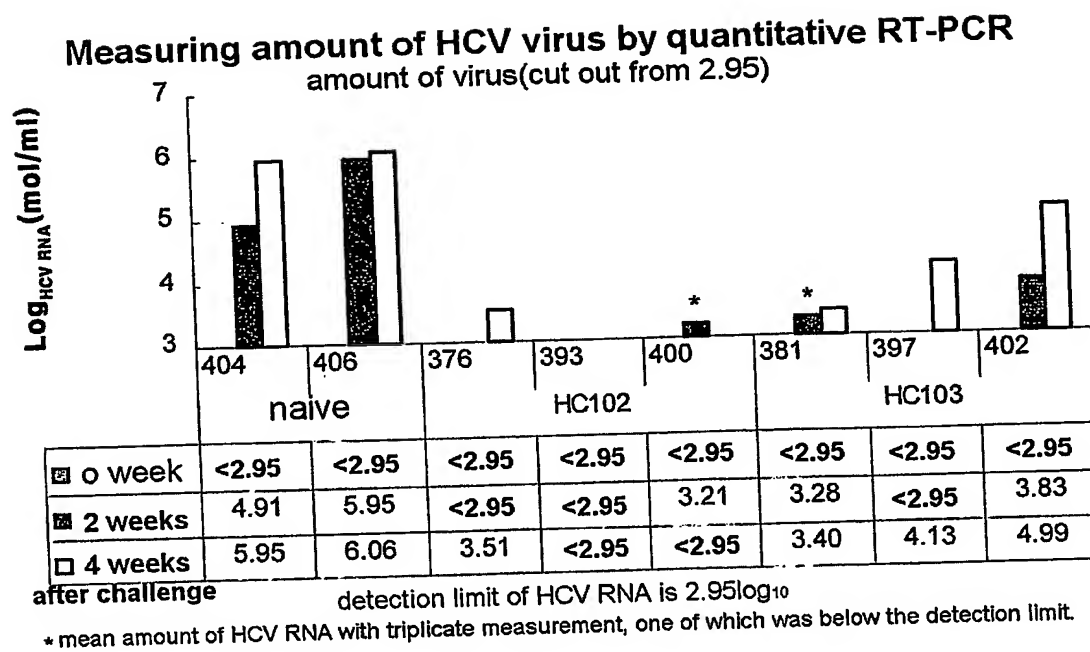
FIG. 18

T cell Proliferative response 2 weeks after rAd boost



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FIG. 19



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FIG. 20a

Amino acid sequence of core peptide pool

ACore (43-191)		
No	Name	Sequence
#1	HCV43-62	RLGURATRKTSERSQPRGRR
#2	HCV53-72	SERSQPRGRR QP IPKARQPE
#3	HCV63-82	QP IPKARQPE GRTWAQPGYP
#4	HCV73-92	GRTWAQPGYP WPLYGNEGLG
#5	HCV83-102	WPLYGNEGLG WAGULLSPRG
#6	HCV93-112	WAGULLSPRG SRPSWGPTDP
#7	HCV103-122	SRPSWGPTDP RRRSRNLGKV
#8	HCV113-132	RRRSRNLGKV IDTLTCGFAD
#9	HCV123-142	IDTLTCGFAD LMGYIPLUGA
#10	HCV133-152	LMGYIPLUGA PLGGUARALA
#11	HCV143-162	PLGGUARALA HGURLLEDGV
#12	HCV153-172	HGURLLEDGV NYATGNLPGC

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FIG. 20b

Amino acid sequence of E2t peptide pool

E2t (384-713)					
No	Name	Sequence	No	Name	Sequence
#13	HCV384-403	STRUTGGTEG RTTHRFVSI	#29	HCV554-573	WMNSTGTFKT CGGPPCDIGG
#14	HCV404-423	ASGPSQRIQL VMNNGSWHIN	#30	HCV564-583	CGGPPCDIGG VGMNTLTCTPT
#15	HCV414-433	VMNNGSWHIN RTALNCNDSL	#31	HCV574-593	VGMNTLTCTPT DCFRKHPEAT
#16	HCV424-443	RTALNCNDSL SSGFIAALFY	#32	HCV584-603	DCFRRHPEAT YTKCGSGPWL
#17	HCV434-453	SSGFIAALFY THKFDSSGCP	#33	HCV594-613	YTKCGSGPWL TPRCHVDYYPY
#18	HCV444-463	THKFDSSGCP ERHASCRIID	#34	HCV604-623	TPRCHVDYYPY RLWHYPCTIN
#19	HCV454-473	ERHASCRIID KFAQGWSIT	#35	HCV614-633	RLWHYPCTIN ITIFKURMYU
#20	HCV464-483	KFAQGWSIT YAESGGSQQR	#36	HCV624-643	ITIFKURMYU GGVEHRLDAA
#21	HCV474-493	YAESGGSQQR PYCWHYAPRQ	#37	HCV634-653	GGVEHRLDAA CNWTRGERCD
#22	HCV484-503	PYCWHYAPRQ CGIUPASQVC	#38	HCV644-663	CNWTRGERCD LEDRDRSELS
#23	HCV494-513	CGIUPASQVC GPVYCITPSP	#39	HCV654-673	LEDRDRSELS PLLLSTTEWQ
#24	HCV504-523	GPVYCITPSP UUUGTIDRSQ	#40	HCV664-683	PLLLSTTEWQ ULPCSETTLP
#25	HCV514-533	UUUGTIDRSQ APTYTGENE	#41	HCV674-693	ULPCSETTLP ALSTGLIHLN
#26	HCV524-543	APTYTGENE TDVLLLNTR	#42	HCV684-703	ALSTGLIHLN QNIVHAQHLN
#27	HCV534-553	TDVLLLNTR PPQANWFGCT	#43	HCV694-713	QNIVHAQHLN GUGSAUUSIU
#28	HCV544-563	PPQANWFGCT WMNSTGTFKT			

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FIG. 20c

Amino acid sequence of NS3 protease peptide pool

NS3 protease (1029-1217)		
#44	gHCV-1029	ITAYSQQTRGLLGCIITSLT
#45	gHCV-1039	LLGCIITSLTGRDKNQVEGE
#46	gHCV-1069	FLATCUNGAWTVFHGAGSK
#47	gHCV-1078	WTVFHGAGSKTLAGPKGPIT
#48	gHCV-1086	TLAGPKGPITQMYTNVDLDL
#49	gHCV-1098	QMYTNVDLDLVGWQAPPGSR
#50	gHCV-1106	VGWQAPPGSRPLTPCTCGSS
#51	gHCV-1118	PLTPCTCGSSDLVLVTRHAD
#52	gHCV-1128	DLVLVTRHADVIPVRRRGDS
#53	gHCV-1138	VIPVRRRGDSRGSLPCPRPV
#54	gHCV-1148	RGSLPCPRPVSYLKGS3GGP
#55	gHCV-1158	SYLKGS3GGPLLCP3GHAUG
#56	gHCV-1168	LLCP3GHAUGIFRAAVCTRG
#57	gHCV-1178	IFRAAVCTRGVAKAUDFIPV
#58	gHCV-1188	VAKAUDFIPVESMETTMRSP
#59	gHCV-1198	ESMETTMRSPVFTDNSTPPA

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FIG. 20d

Amino acid sequence of Helicase peptide pool

NS3 helicase (1208-1656)					
No	Name	Sequence	No	Name	Sequence
#60	HCV1208-1227	VITDNSTPPA UPQTFQVAHL	#77	HCV1458-1477	TQTUDFSLDP TIT IDTTTUP
#61	HCV1218-1237	UPQTFQVAHL HAPTGSCKST	#78	HCV1468-1487	TIT IDTTTUP QDAUSRSQRR
#62	HCV1228-1247	HAPTGSCKST KUPAAYAAQG	#79	HCV1478-1497	QDAUSRSQRR GRTGRGRRGI
#63	HCV1238-1257	KUPAAYAAQG YKULULNPSV	#80	HCV1488-1507	GRTGRGRRGI YRFVTPGERP
#64	HCV1248-1267	YKULULNPSV AATLGFGVYM	#81	HCV1498-1517	YRFVTPGERP SGMDSSULC
#65	HCV1258-1277	AATLGFGVYM SKAHGIDPHI	#82	HCV1518-1537	ECYDAGCAWY ELTPAETSUR
#66	HCV1268-1287	SKAHGIDPHI RTGURAITTG	#83	HCV1528-1547	ELTPAETSUR LRAYLNTPLG
#67	HCV1278-1297	RTGURAITTG APITYSTYCK	#84	HCV1538-1557	LRAYLNTPLG PUCQDHLEFW
#68	HCV1318-1337	HSTDSTSILG IGTULDQAEI	#85	HCV1548-1567	PUCQDHLEFW ESUFTGLTHI
#69	HCV1328-1347	IGTULDQAEI AGARLUULAT	#86	HCV1558-1577	ESUFTGLTHI DAHILSQTQK
#70	HCV1348-1367	ATPPGSVTUP HPNIEEVALS	#87	HCV1568-1587	DAHILSQTQK AGDNFPYLVA
#71	HCV1358-1377	HPNIEEVALS NTGEIPFYCK	#88	HCV1578-1597	AGDNFPYLVA YQATUCARAQ
#72	HCV1368-1387	NTGEIPFYCK AIP IEVIRGG	#89	HCV1588-1607	YQATUCARAQ APPPSWDQHW
#73	HCV1388-1407	RHLIFCHSKK KSDLAALKS	#90	HCV1598-1617	APPPSWDQHW KCLTRLKPTL
#74	HCV1398-1417	KSDLAALKS ALGLNAVAYY	#91	HCV1608-1627	KCLTRLKPTL HGPTPLLYRL
#75	HCV1408-1427	ALGLNAVAYY RGLDUSVIPT	#92	HCV1618-1637	HGPTPLLYRL GAUQNEUTLT
#76	HCV1418-1437	RGLDUSVIPT SGDUUUVATD	#93	HCV1628-1647	GAUQNEUTLT HPVTKFIMAC

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FIG. 20e

Amino acid sequence of NS5A peptide pool

NS5A (1972-2411)					
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#95	gHCV-1982	WICTULTDFKTWLSKLLPR	#114	gHCV-2202	ASQLSAPSLKATCTIHNDSP
#96	gHCV-1992	TWLSKLLPRLPGVFFSCQ	#115	gHCV-2212	ATCTIHNDSPDADLIEANLL
#97	gHCV-2002	LPGVFFSCQRGKGVWRGE	#116	gHCV-2222	DADLIEANLLWRQEMGGNIT
#98	gHCV-2012	RGYKGVWRGEGIMQTTCPGG	#117	gHCV-2232	WRQEMGGNITRUESENKVI
#99	gHCV-2022	GIMQTTCPGGAQIAGHVWNG	#118	gHCV-2242	RUESENKVIILDSFEPIRAE
#100	gHCV-2042	SMRIUGPRTCSNTWNGTIP I	#119	gHCV-2252	LDSFEP IRAEEDEREVSUPA
#101	gHCV-2052	SNTWNGTIP INAYTTGPCSP	#120	gHCV-2262	EDEREVSUPAEILRRSRKFP
#102	gHCV-2062	NAYTTGPCSPSPAPNYSRAL	#121	gHCV-2272	EILRRSRKFPAMP IWARPD
#103	gHCV-2072	SPAPNYSRALWRVAAEEYVE	#122	gHCV-2292	YMPPLLESUKDPDYUPPUH
#104	gHCV-2082	WRVAAEEYVEUTRUGDFHYU	#123	gHCV-2302	DPDYUPPUHGCPLPPTKAA
#105	gHCV-2092	UTRUGDFHYUTGUTTDNUKC	#124	gHCV-2322	PIPPPRRKRTIULTTESTUSS
#106	gHCV-2102	TGUTTDNUKCPQUPAPEFF	#125	gHCV-2332	IULTTESTUSSALAEATKTF
#107	gHCV-2122	TELDGURLHRYAPACKPLLR	#126	gHCV-2342	ALAEATKTFGGSGSWAADS
#108	gHCV-2132	YAPACKPLLRDEVSFQUGLN	#127	gHCV-2352	GGSGSWAADSGTATAPPDQT
#109	gHCV-2152	QYLUGSQLPCEPEPDVAULT	#128	gHCV-2372	SDDGDKESDUESYSSEPPLE
#110	gHCV-2162	EPEPDVAULTSHLTDP SHIT	#129	gHCV-2382	ESYSSEPPLEGEPEGDPDLSD
#111	gHCV-2172	SHLTDP SHIT AETAKRRLAR	#130	gHCV-2392	GEPEGDPDLSDGSUSTUSEEA
#112	gHCV-2182	AETAKRRLARGSPPSLASSS			

SEQUENCE LISTING

<110> Genexine Inc.
POSTECH FOUNDATION
DONG-A PHARM. CO., LTD.
DAEWOONG CO., LTD.
POSCO

<120> A vaccine enhancing the protective immunity to Hepatitis C virus using plasmid DNA and recombinant adenovirus

<130> 3fpo-08-04

<150> KR 2002-58712
<151> 2002-09-27

<150> KR 2002-68496
<151> 2002-11-06

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DNA
Artificial Sequence

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PCR primer

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<211> 2178

<212> DNA

<213> Hepatitis C virus and Herpes Simplex Virus (gDsΔST)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01951

A. CLASSIFICATION OF SUBJECT MATTER		
IPC7 A61K 39/29		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC7 A61K 39/29		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Korean patents and applications for inventions since 1975		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA, NCBI pubmed database, NCBI blastn database, Delphion Research Intellectual Property network database		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Brinster C et al. 'Hepatitis C virus non-structural protein 3-specific cellular immune responses following single or combined immunization with DNA or recombinant Semliki Forest virus particles.' In J Gen Virol., 2002 Feb, vol.83(Pt 2), p369-81 see the whole document	1-29, 30-37
A	Brinster C et al. 'Different hepatitis C virus nonstructural protein 3 (Ns3)-DNA-expressing vaccines induce in HLA-A2.1 transgenic mice stable cytotoxic T lymphocytes that target one major epitope.' In Hepatology, 2001, vol.34, No.6, p1206-17. see the whole document	1-29, 30-37
A	Lazdina U et al. 'Humoral and CD4(+) T helper (Th) cell responses to the hepatitis C virus non-structural 3 (NS3) protein: NS3 primes Th1-like responses more effectively as a DNA-based immunogen than as a recombinant protein.' In J Gen Virol, 2001, vol. 82, Pt 6, p1299-308. see the whole document	1-29, 30-37
A	Forns X et al. 'Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV.' In Hepatology, 2000, vol.32, No.3, p618-25. see the whole document	1-29, 30-37
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search 10 DECEMBER 2003 (10.12.2003)		Date of mailing of the international search report 11 DECEMBER 2003 (11.12.2003)
Name and mailing address of the ISA/KR  Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140		Authorized officer LIM, Hea Joon Telephone No. 82-42-481-5590 

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01951

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nagayama K et al, 'Time-related changes in full-length hepatitis C virus sequences and hepatitis activity.' In Virology, 1999, Vol.263, No.1, p244-253 (NCBI accession No:AF165060) see the whole document	1-29, 30-37
A	NCBI accession No:AF207765, 27-APR-2000	1-29, 30-37
A	NCBI accession No:D14484, 03-FEB-1999	1-29, 30-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01951

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 39/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 A61K 39/29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, NCBI pubmed database, NCBI blastn database, Delphion Research Intellectual Property network database

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☐ See patent family annex.

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"&" document member of the same patent family


Date of the actual completion of the international search

10 DECEMBER 2003 (10.12.2003)

Date of mailing of the international search report

11 DECEMBER 2003 (11.12.2003)

Name and mailing address of the ISA/KR


Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer

LIM, Hea Joon

Telephone No. 82-42-481-5590



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01951

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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